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# United States Patent [19]

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[54] **AUTOMATED ANALYSIS EQUIPMENT AND ASSAY METHOD FOR DETECTING CELL SURFACE PROTEIN FUNCTION USING SAME**

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[\*] Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2). This patent is subject to a terminal disclaimer.

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[21] Appl. No.: **08/229,150**

[22] Filed: **Apr. 18, 1994**

### Related U.S. Application Data

[63] Continuation of application No. 07/812,254, Dec. 20, 1991, abandoned.

[51] **Int. Cl.**<sup>7</sup> ..... **G01N 33/53**; G01N 33/566; G01N 15/06; C12P 21/06

[52] **U.S. Cl.** ..... **435/7.2**; 435/7.1; 435/69.1; 436/501; 436/43; 436/79; 436/800; 422/68.1; 422/82.08

[58] **Field of Search** ..... 422/62, 68.1, 82.08; 435/7.2, 7.21, 69.1, 79; 436/43, 79, 501, 800

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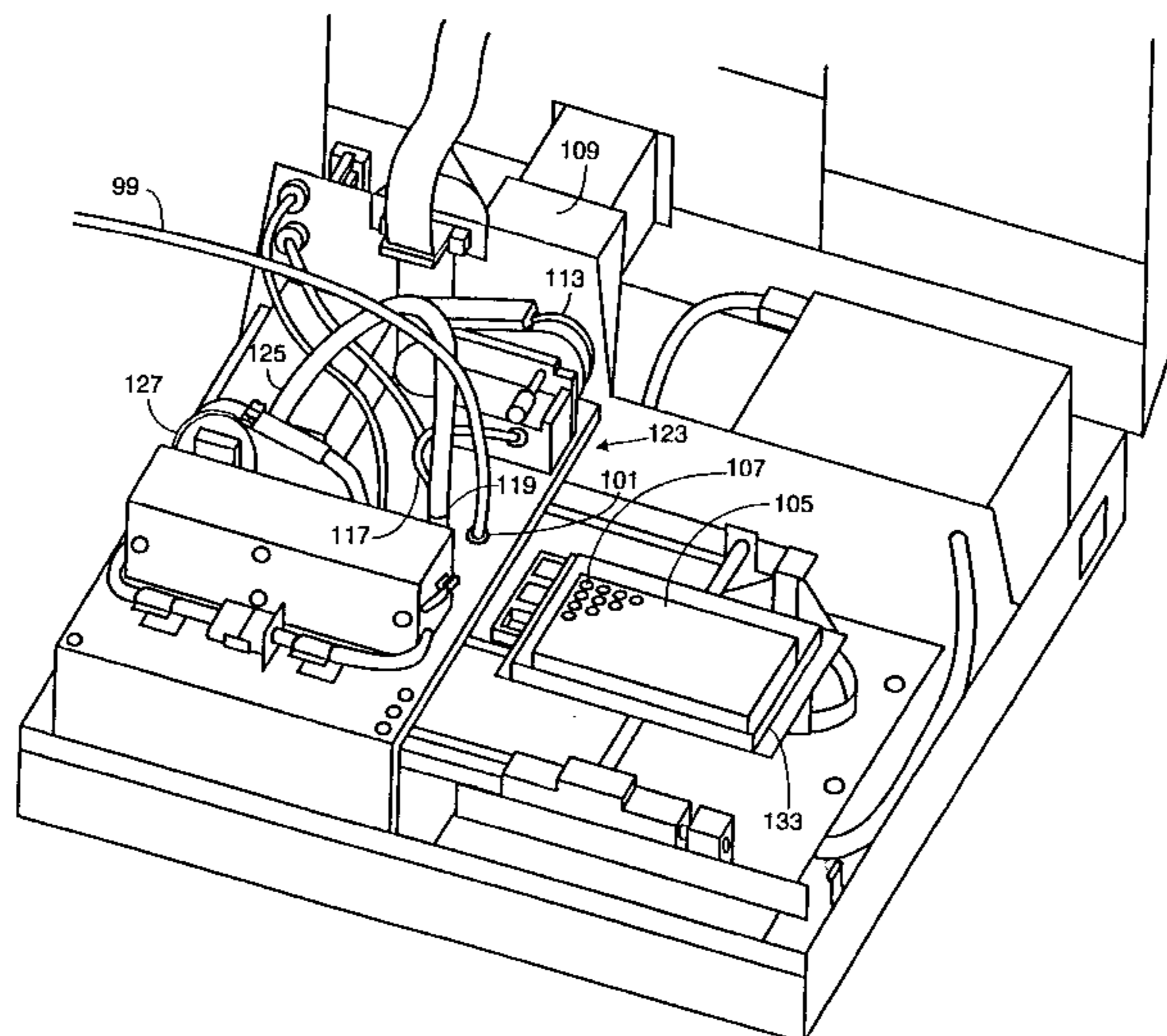
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### [57] ABSTRACT

Automated drug screening method and a method of studying receptor and ion-channel activity are provided.

**43 Claims, 7 Drawing Sheets**



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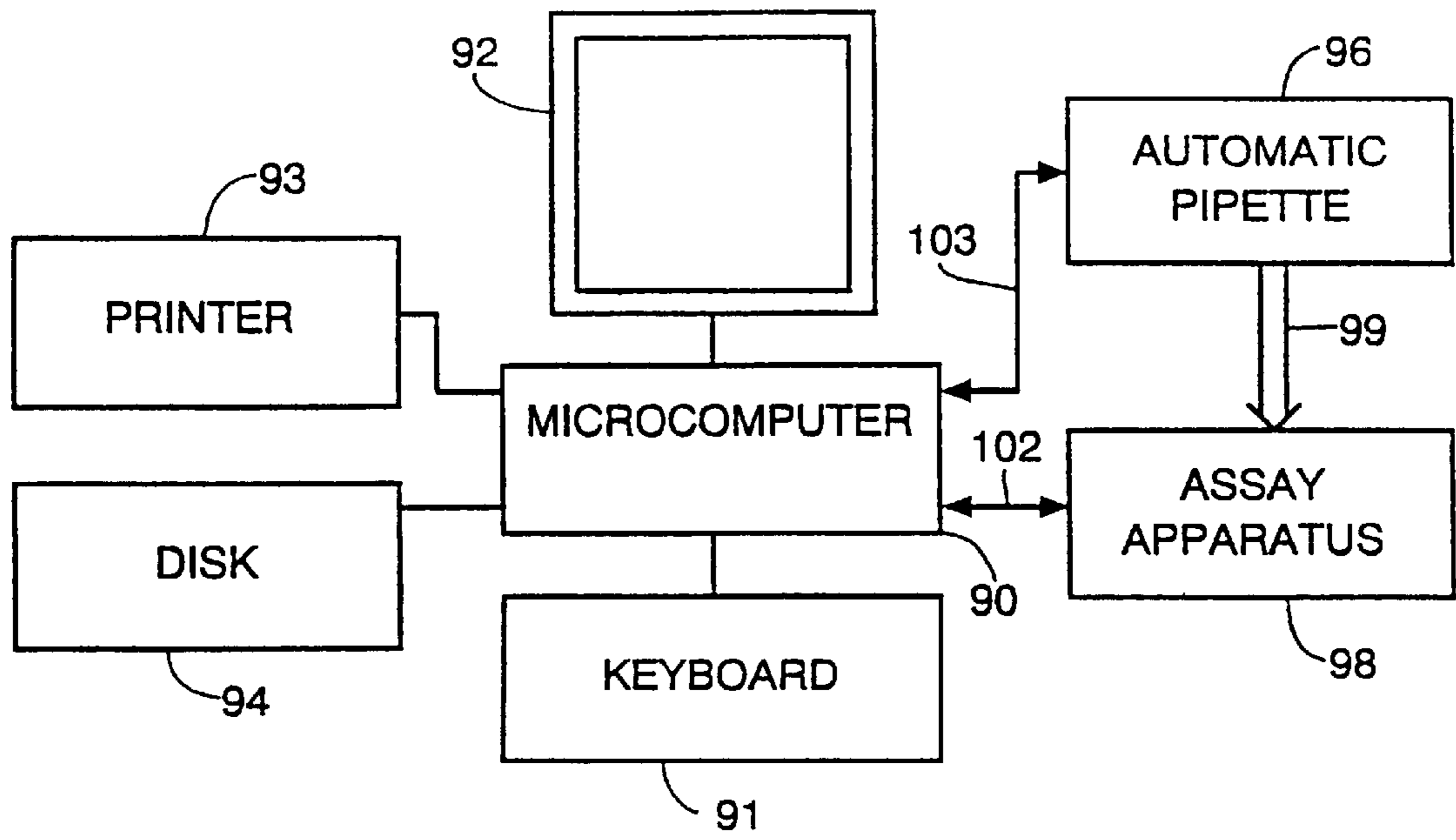


Fig. 1

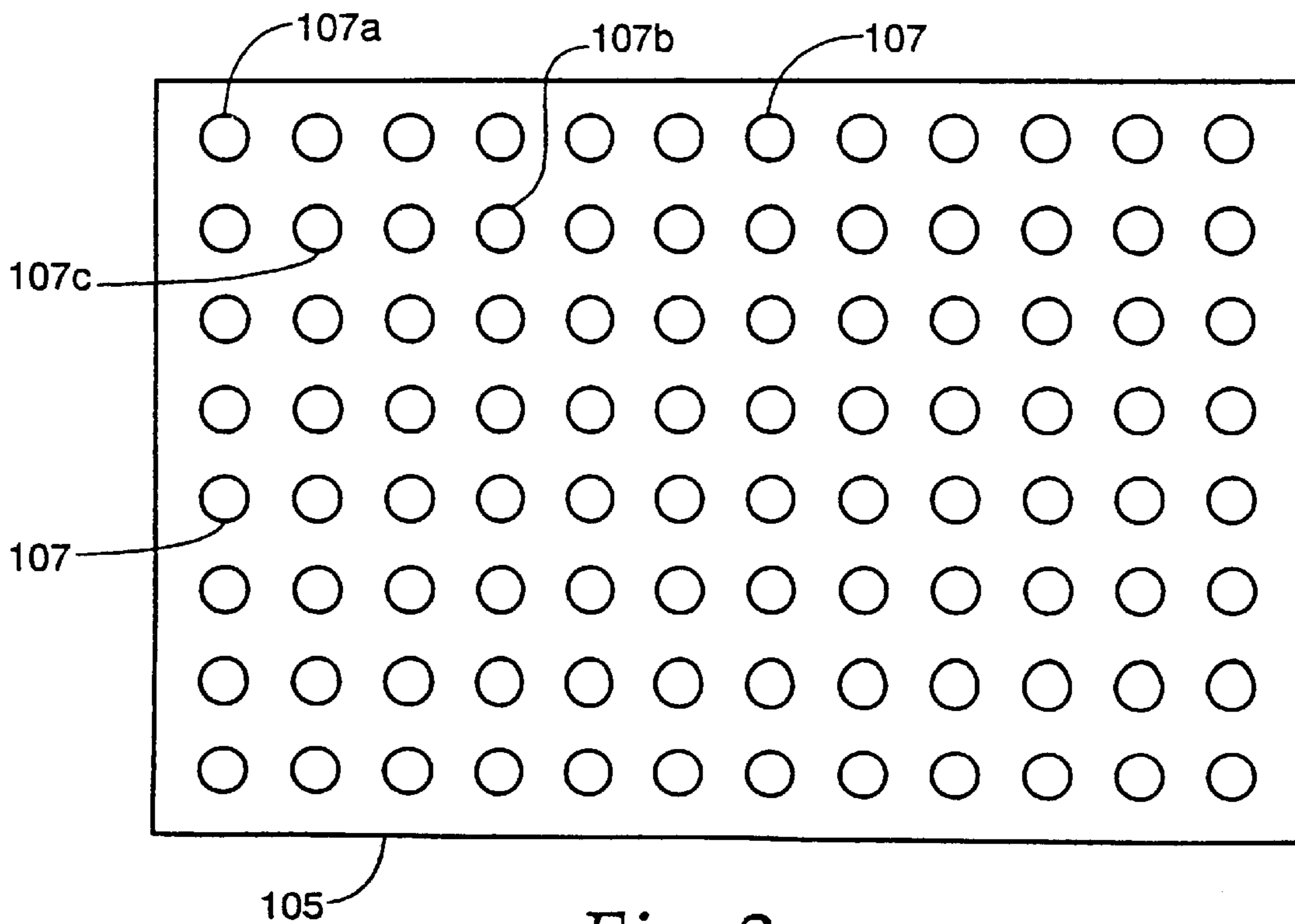


Fig. 2

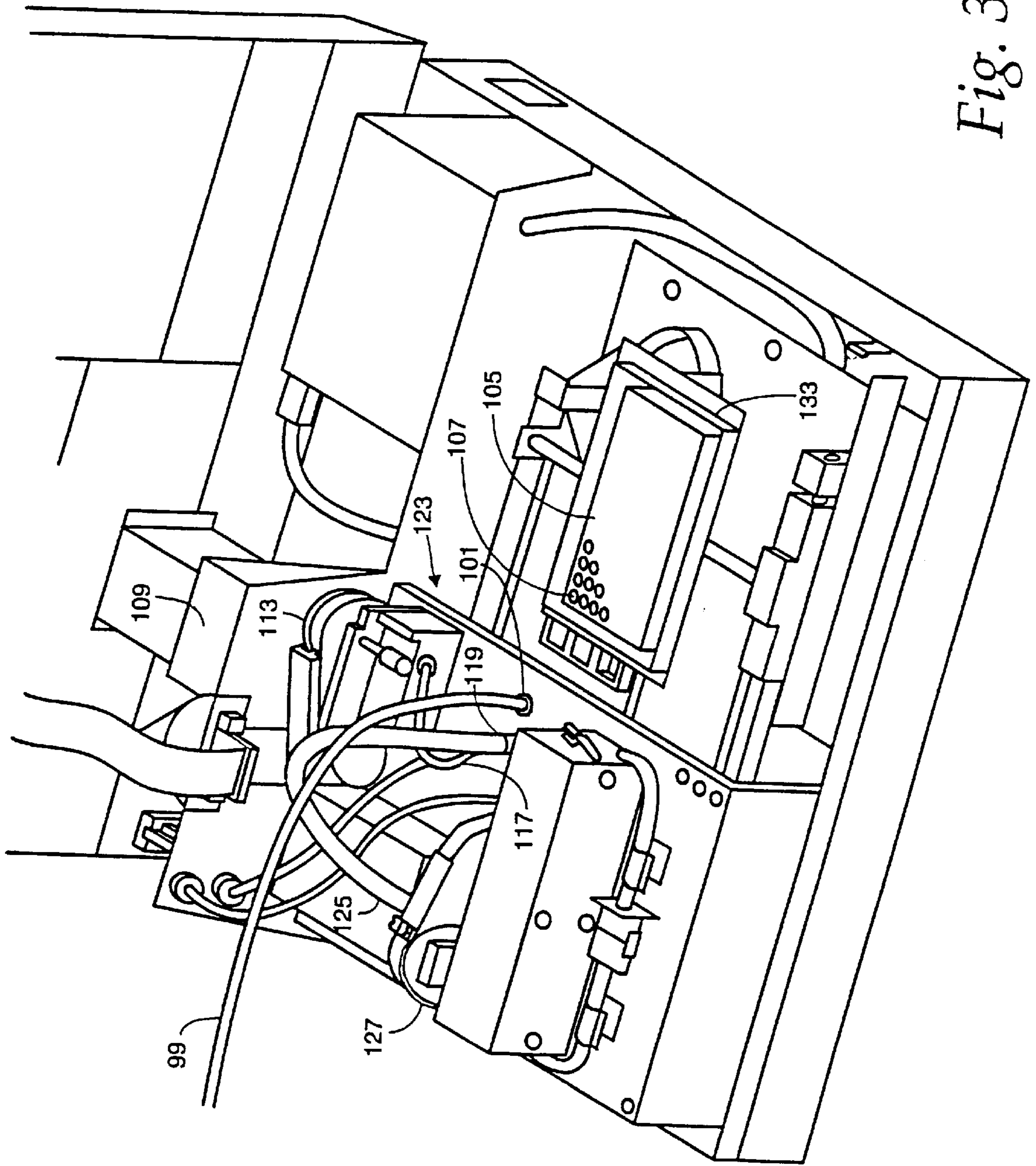


Fig. 3

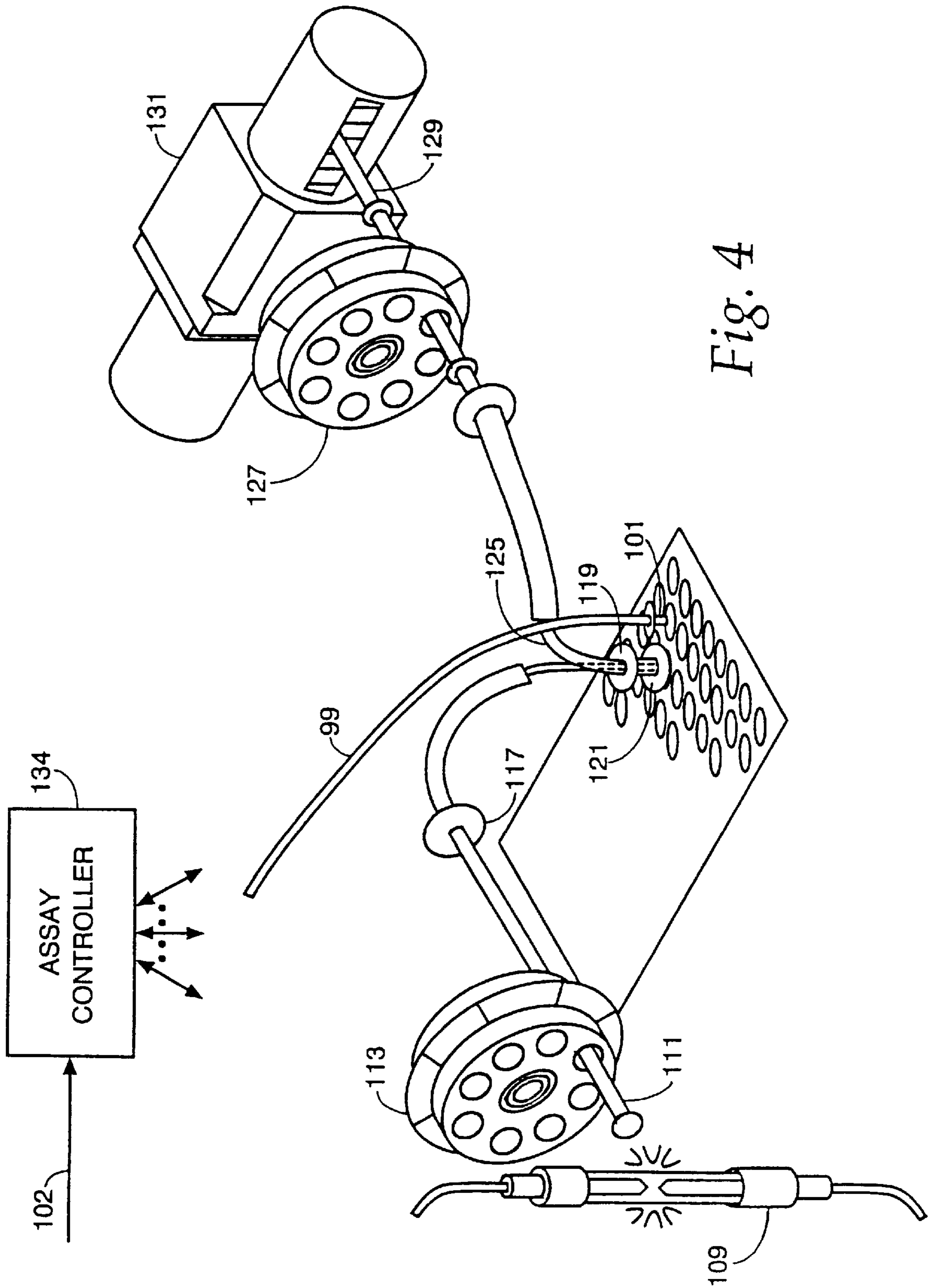


Fig. 4

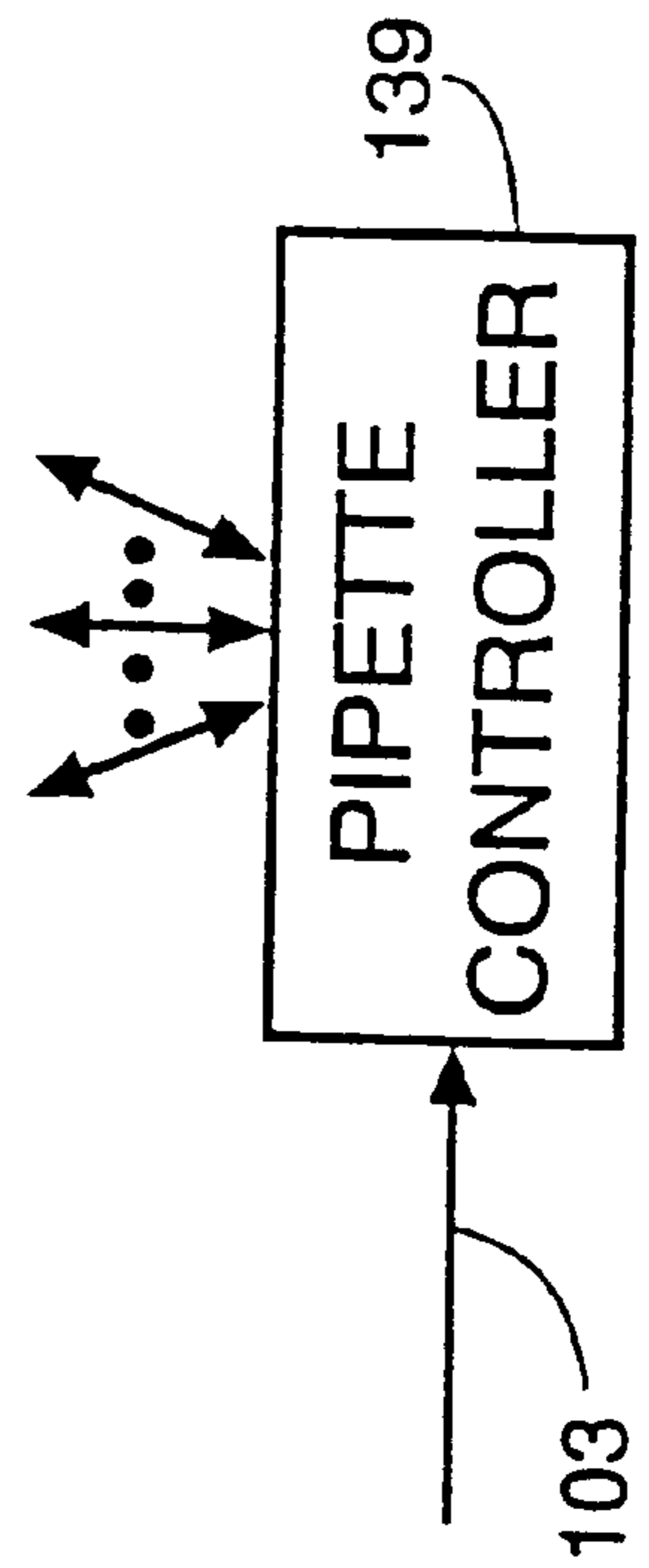
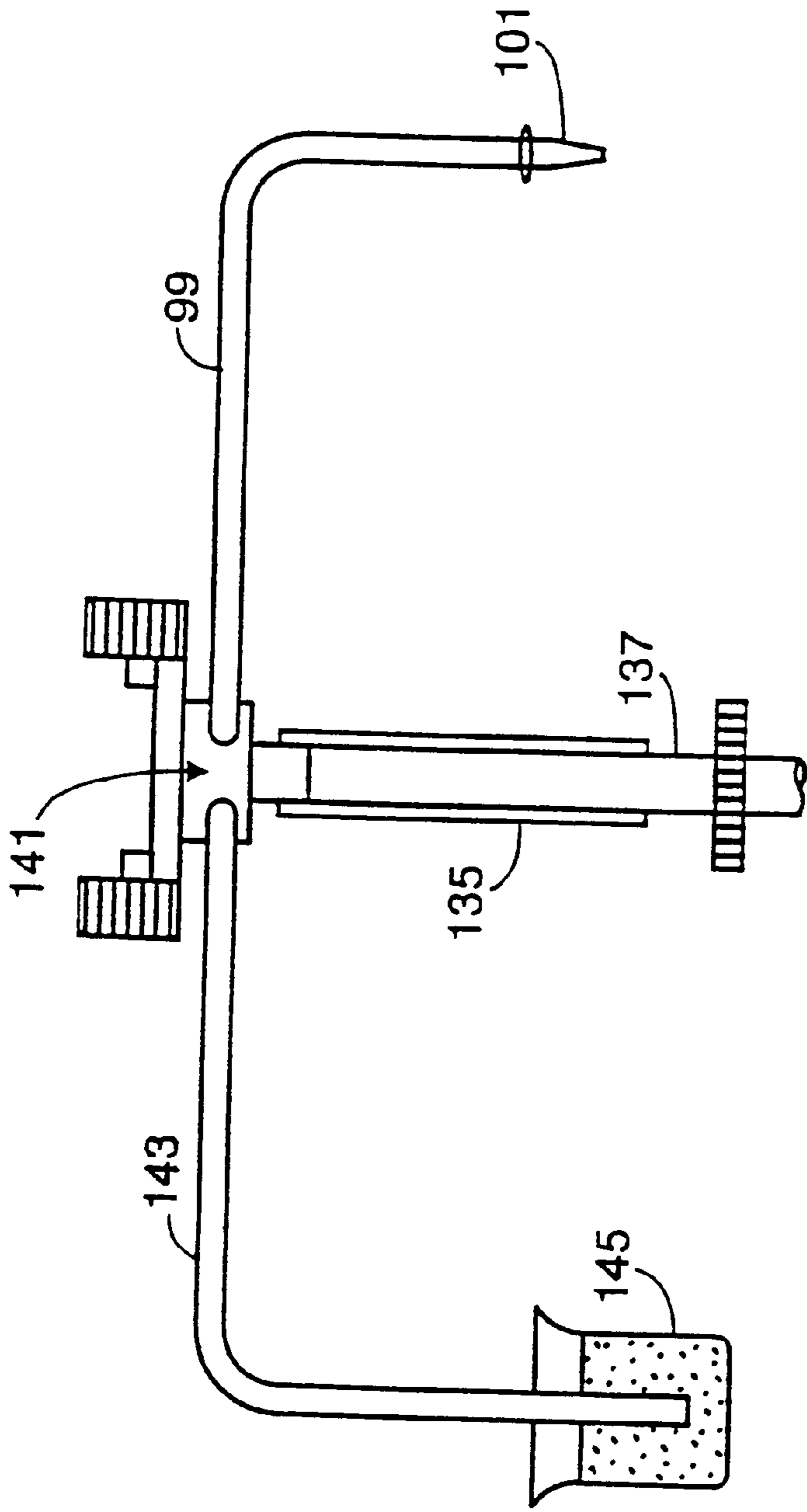


Fig. 5

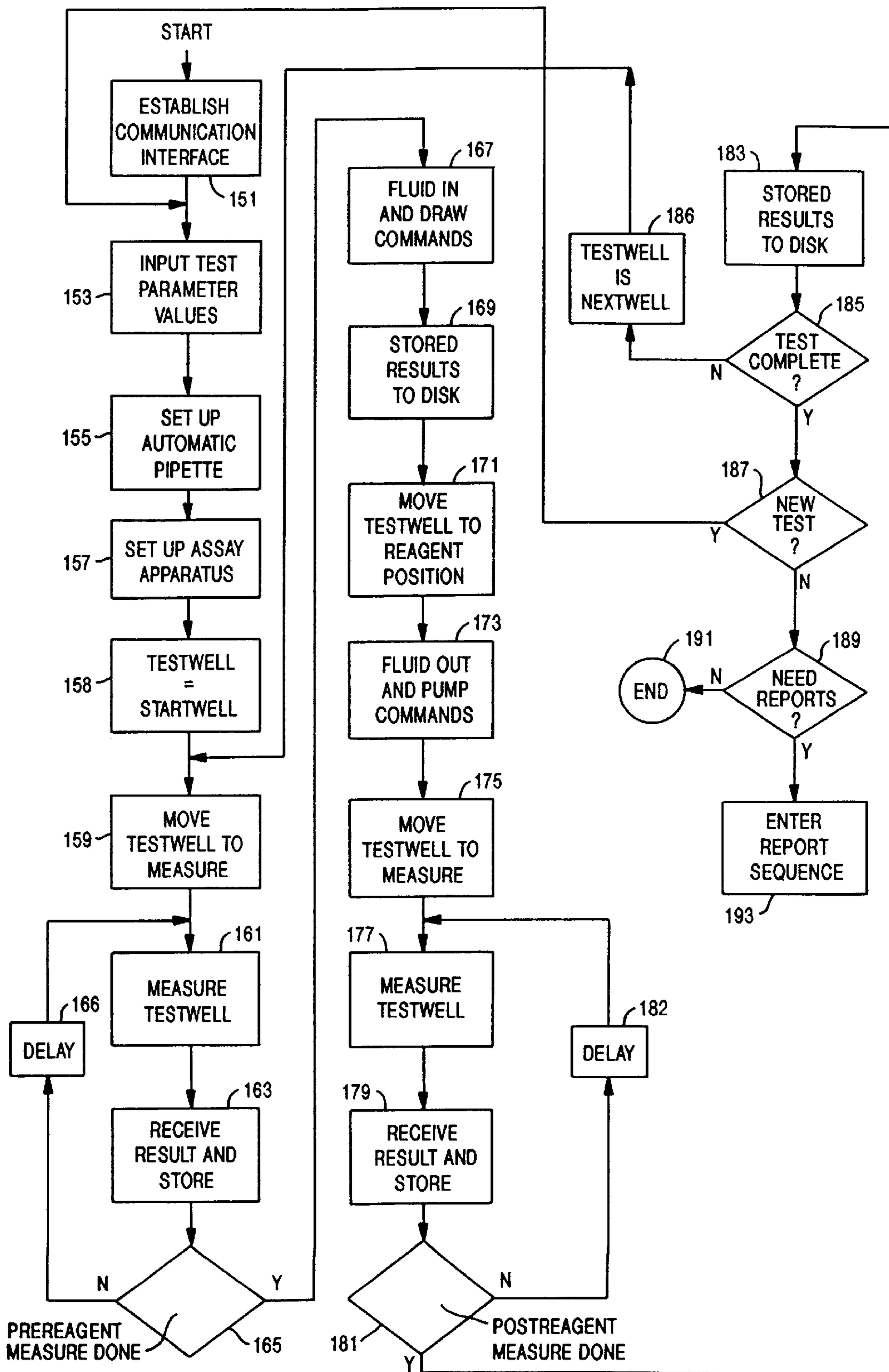


Fig. 6

START WELL	3, 3
NUMBER OF WELLS	8
REAGENT QUANTITY DRAW	50 $\mu$ l
REAGENT QUANTITY PUMP	50 $\mu$ l
EXCITATION FILTER	ex2
EMISSION FILTER	em2
PRE-REAGENT READS	5
POST-REAGENT READS	125
DATA FILE NAME	TEST1
SYRINGE SPEED	W2

*Fig. 7*



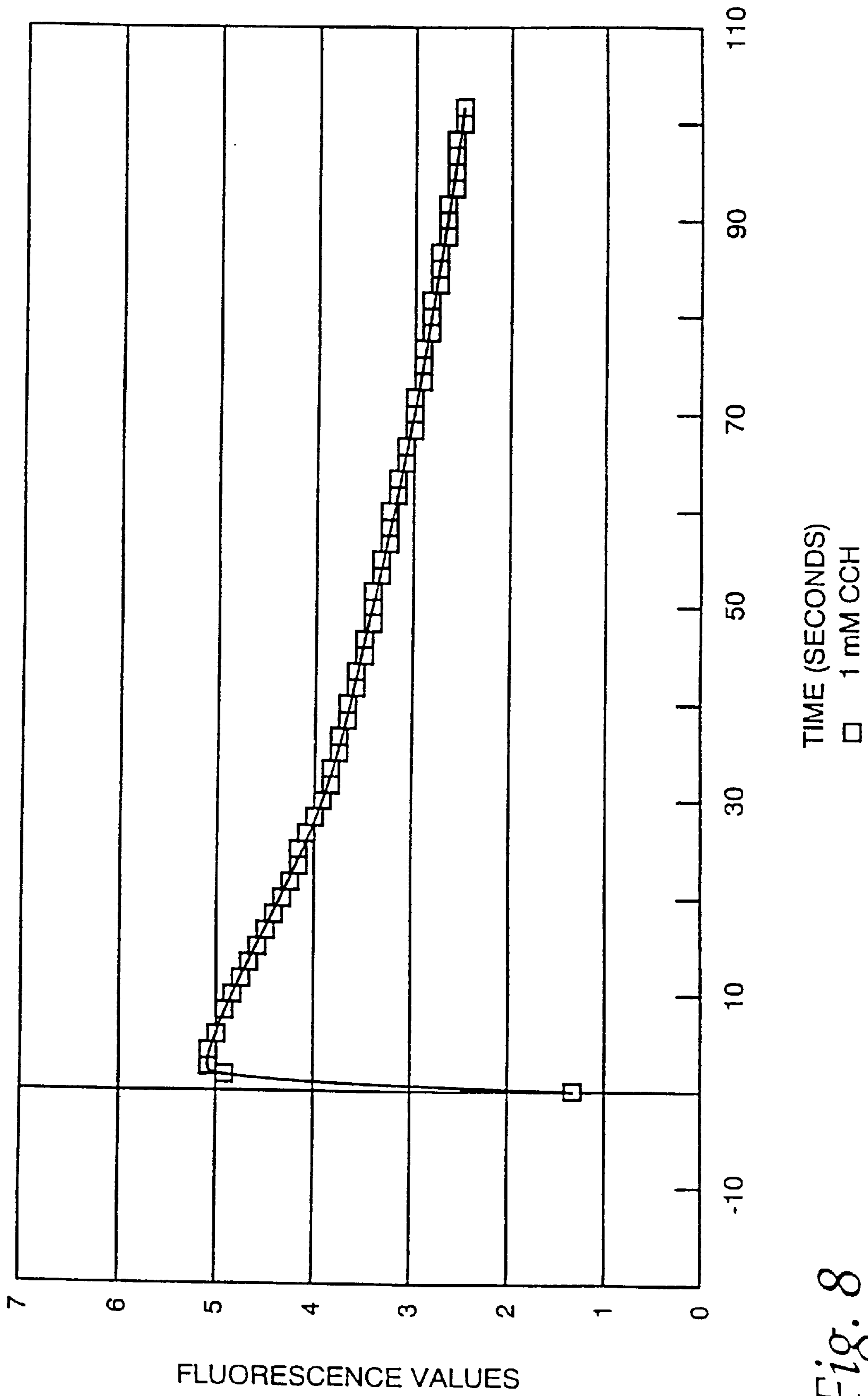


Fig. 8

**AUTOMATED ANALYSIS EQUIPMENT AND  
ASSAY METHOD FOR DETECTING CELL  
SURFACE PROTEIN FUNCTION USING  
SAME**

This is a continuation of application Ser. No. 07/812,254, filed Dec. 20, 1991, now abandoned.

**BACKGROUND OF THE INVENTION**

This invention relates to methods and apparatus for assaying chemical samples to which a reagent is added and particularly, to computer-controlled methods and apparatus for such assaying.

Assaying processes are well known in which a reagent is added to a sample, and measurements of the sample and reagent are made to identify sample attributes stimulated by the reagent. For example, one such assay process concerns determining in a chromogenic assay the amount of an enzyme present in a biological sample or solution. Such assays are based on the development of a colored product in the reaction solution. The reaction develops as the enzyme catalyzes the conversion of a colorless chromogenic substrate to a colored product.

In such assays it is often required to determine the enzymatic activity of a number of samples and at one or more dilutions. Enzymatic reactions characteristically proceed at a constant rate provided substrate is present in a large molar excess, i.e., the concentration of substrate does not limit the rate of reaction. With such kinetic parameters, it may be convenient to set up several reaction solutions separately in the wells of a microtiter plate, for example, carrying out each reaction for a predetermined constant amount of time and stopping the reactions while they are still in a linear range of the assay. With each of the so-called end-point reactions stopped, no further color development occurs and the reaction solutions in the separate wells of the microtiter may be read at any convenient time.

Plate readers which automatically read the intensity of a colored solution in an array of wells are known. Also, plate readers which measure the amount of fluorescence in a well of a microtiter plate are known.

Classically, assays of the above-described type are performed by a laboratory worker who prepares the sample, manually adds a precise amount of reagent to the sample, and then measures the result at one or more preselected times after the reagent addition. This classical approach is very time consuming for the laboratory worker and additionally, when the stimulated reaction yields time-varying results, precise timing on the part of the laboratory worker is required. If such timing is not properly performed, erroneous assay results may occur.

One known laboratory device for partially automating tests to detect fluorescence as a measurable attribute is the Fluoroskan II. The Fluoroskan II includes a plate carrier system to hold a sample-containing plate having a plurality, e.g., 96, of sample-containing wells. A laboratory worker places a portion of the sample into some or all of the wells of the plate, and then adds reagent to the sample-containing wells. The plate is then placed in the Fluoroskan II which automatically measures the fluorescence of the samples in the wells. Although this known apparatus has proven valuable for fluorescence testing, some problems, which were also inherent in the classical testing, still remain. For example, the addition of reagent by the laboratory worker still requires a large amount of laboratory work time. Also, since the measured results of reactions may be dependent on

the time elapsed since reagent addition, the first samples to receive reagent may have progressed past the point of meaningful reaction results by the time all samples have received reagent. Further, some reactions complete so quickly that it is nearly impossible to add reagent to a sample, move the sample plate to the assay apparatus and move the sample to a measurement position before the reaction has run to completion.

A need exists to rapidly screen compounds to determine their effect on a protein's function such as cell surface proteins like ion channels and receptors, the regulation of which surface proteins can be important in treating certain disease states. Such cell surface proteins permit intracellular transduction of extracellular signals. These cell surface proteins, by transmitting information regarding extracellular stimuli via specific intracellular pathways, induce an appropriate cellular response to external stimuli. The response may be immediate and transient, slow and sustained, or some mixture thereof. By virtue of an array of varied membrane surface proteins, normal (i.e., non-diseased) cells and tissue are exquisitely sensitive to their environment. Compounds which are capable of potentiating or inhibiting activation of voltage-dependent calcium channels are believed to be useful in treating a variety of diseases including certain cardiovascular and nervous system disorders. Similarly, compounds which can affect the functioning of cell surface receptors may be beneficial in the treatment of certain other diseases. Thus, it is desirable to identify compounds capable of potentiating or inhibiting such cell surface proteins using functional assays. Such assays require study of the kinetics of the reaction in real time due to their transient nature. Heretofore, such assays have required a substantial amount of time from highly skilled researchers and technicians to conduct and record the results of individual assays. Also, with respect to functional assays for cell surface proteins which utilize electrophysiological or fluorescence imaging techniques, laboratory equipment which is required is often extremely costly.

A need also exists for an automated analysis apparatus which is capable of automatically adding reagent to contained samples and measuring reaction results to minimize lab worker time requirements, and to provide accurate measurement of time varying reactions. It would also be desirable to be able to provide a rapid automated method for assaying a compound or a series of compounds for ability to affect cell surface proteins such as ion channels and receptors where a plurality of tests may be conducted seriatim and the results recorded with no, or only occasional, intervention by the researcher.

**SUMMARY OF THE INVENTION**

This need is met and a technical advance is achieved with the present invention, which in one aspect is a computer-controlled measurement apparatus for automatically adding reagent to a predetermined sample-containing well and measuring at least one attribute of the sample in the predetermined well. The computer-controlled measurement apparatus comprises a plate having a plurality of solution-containing wells, reagent-adding equipment responsive to the computer for adding reagent to the wells, measurement equipment for measuring at least one attribute, e.g., fluorescence, of the solution contained by the wells and moving equipment which is responsive to the computer for aligning one of the wells first with the reagent-adding component, then with the fluorescence measurement device.

The computer exercises control over the operation of the various components involved in order to properly coordinate

the functions of the apparatus: reagent addition and attribute measurement. The computer issues several types of commands to provide the necessary coordination. In one embodiment, alignment of wells with reagent-adding and fluorescence-measuring devices is accomplished by computer-issued plate movement commands which are sent to the plate-moving equipment which responds by moving a predetermined well to the reagent-adding position, then to the measurement position. Pump commands are generated by the computer to direct the reagent-adding equipment to add a predetermined volume of the reagent to the predetermined well at the reagent-adding position. Additionally, measurement commands are generated by the computer to direct the measuring equipment to measure a plurality of fluorescence magnitude values of the sample in the predetermined well. The values measured by the measurement equipment are recorded within the assay apparatus for later use. In the preferred embodiment, the measured values are first stored within a microcomputer, which comprises the controller, and later moved to a disk drive for long-term storage.

In the performance of the present assay, the controller first aligns a predetermined well containing a sample to be assayed with a reagent-adding position, then controls the reagent-adding equipment to add a predetermined volume of reagent to the predetermined well while it is in the reagent-adding position. After reagent is added, the predetermined well is aligned, under the control of the controller, with a measurement position. The fluorescence of the sample in the predetermined well is measured using a filter and a photomultiplier tube to detect emitted light, again in response to computer control, while the predetermined well is aligned with the measurement position. Advantageously, the measurement equipment comprises a light source and filters for stimulating fluorescence and a photomultiplier tube to detect light emitted by the sample in the predetermined well using fiber optic cables to send and receive light.

In certain situations, the sample contained by a well may exhibit background levels of the attribute to be measured, e.g., fluorescence, even before reagent is added to the well. In such cases, it is desirable to measure the attribute prior to adding reagent so that a background (pre-reagent) value can be used in data analysis to more accurately evaluate the measured post-reagent values. Accordingly, for certain tests the predetermined well is aligned, under the control of the controller, with the measuring equipment, and pre-reagent measurements are taken and stored.

A method of operation of a computer-controlled fluorescence-measuring apparatus comprises identifying a predetermined well to be measured, moving the predetermined well to a reagent-adding position, and adding reagent to the predetermined well while at the reagent-adding position. After adding reagent, the predetermined well is moved to a measurement position where the fluorescence of the sample in the predetermined well is measured and the data from such measurement recorded. In certain situations, the pre-reagent fluorescence of the sample in the predetermined well may be measured by moving the predetermined well to the measuring position and measuring fluorescence values prior to the adding of reagent. The fluorescence measurement of the method may also be performed by automatically identifying a plurality of wells one at a time as the well to be measured and performing the method on each of the identified plurality of wells.

Also, an automated method is provided for testing the response of a cell having cell surface ion channels or receptors to one or more compounds having putative ion

channel or receptor modulatory activity, where the ion channels or receptors of the cells, when activated, are capable of directly or indirectly causing an increase in the concentration of ions in the cytoplasm, and wherein the degree of activation or inactivation of the ion channels or receptors is determined by development or lack of development of fluorescence in the cytoplasm of the cells which cells have been loaded with an amount of an ion-sensitive fluorescent dye sufficient to detect a change in intracellular ion concentration.

In a particular aspect, the invention provides an automated method for rapid functional screening of compounds to identify potential pharmaceuticals, i.e., drugs. For the first time, an efficient drug-screening method is provided which utilizes the computer-controlled fluorescence-measuring apparatus described above for rapid automated analysis of one or more compounds that is based on functional evaluation of drug targets, i.e., receptors and ion channels, in their physiological environment, i.e., living cells, in the presence of the potential pharmaceuticals. In the performance of the drug-screening assay, the sample wells contain receptor- and/or ion channel-expressing cellos, the test compound is either delivered to the wells via the reagent-adding device (in the case of an agonist-like compound) or present in the well (in the case of an antagonist-like compound), and the fluorescence of the cells is measured by the fluorescence-measuring device. Because many cells can be assayed in a relatively short period of time, the present invention enables rapid analysis of replicate samples, including control samples, and provides a possibility for screening multiple compounds and/or multiple doses of compounds in a single operation. In an especially preferred embodiment, the cells are recombinant cells expressing a homogeneous population of recombinant receptors in channels thereby providing an assay which is valuable for determining the specificity of a compound having putative agonist or antagonist activity with respect to the receptors or ion channels.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a block diagram of an automated apparatus embodying the invention;

FIG. 2 is a plan view of a multi-well plate used with an assay apparatus of FIG. 1;

FIG. 3 is a perspective view of the internal structure of the assay apparatus of FIG. 1;

FIG. 4 is a diagrammatic perspective view of the structure of the assay apparatus emphasizing portions thereof;

FIG. 5 is a diagrammatic view of the structure of an automatic pipette of the embodiment of FIG. 1;

FIG. 6 is a flow diagram of a program implemented by a microcomputer to coordinate the performance of assays by the embodiment of FIG. 1;

FIG. 7 represents a monitor screen displayed list of test parameters and values assigned thereto; and

FIG. 8 shows a report of fluorescence values measured by the automated apparatus of FIG. 1.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

FIG. 1 is a block diagram of a system embodying the present invention. The system includes a microcomputer 90 connected in the conventional manner to a keyboard 91, a monitor 92, a printer 93 and a disk drive 94. In the preferred embodiment, microcomputer 90 is an IBM or IBM-compatible microprocessor (e.g., a microcomputer com-

monly known as an IBM PC). Assays are performed by cooperative interaction of an automatic pipette 96 and an assay apparatus 98, which are controlled by microcomputer 90 via a respective one of bi-directional buses 103 and 102. In the preferred embodiment, communication over buses 102 and 103 is in the RS-232C format. During an assay, automatic pipette 96, at times and in amounts controlled by microcomputer 90, pumps reagent to assay apparatus 98 via tubing 99. The assay apparatus 98 is used to individually measure fluorescence of solutions contained by a plurality of solution wells. Under control of microcomputer 90, the solution in a given well receives the predetermined quantity of reagent from automatic pipette 96 and the fluorescence of the solution in that well is measured by assay apparatus 98 and recorded in the microcomputer 90 for later analysis.

The quantities of solution are presented to the assay apparatus 98 for measurement in a multi-well plate 105 as shown in plan view in FIG. 2. Multi-well plate 105 comprises 96 recesses or wells 107, each for receiving an individual quantity of solution for analysis. The wells 107 are regularly spaced to form a rectangular array having eight rows of wells 107 with each row comprising 12 columns of wells. Each well has a specific X, Y location in the array with the variable X denoting one of the 12 columns, and the variable Y denoting one specific well within the column identified by the X variable. For example, the well denoted 107a in FIG. 2 is identified as well 1,1 while the well denoted 107b is identified by as well 4,2. In preparation for analysis, solutions to be tested are placed in some or all of the wells 107 and the multi-well plate 105 is placed in assay apparatus 98.

FIG. 3 is a perspective view of the internal components of assay apparatus 98 showing a multi-well plate 105 therein. The assay apparatus 98 in the present embodiment is a modified version of the Fluoroskan II manufactured by Labsystems of Helsinki, Finland (Sold in the United States by Lab Products International, Raleigh, N.C.). The operation of the Fluoroskan II and its interaction with a microcomputer are well known in the art and are described for example in the Fluoroskan II operating instructions No. 1500750. Portions of the assay apparatus 98 shown in FIG. 3 are also shown in a selective diagrammatic perspective view in FIG. 4 for ease of understanding.

Assay apparatus 98, exposes the samples in individual wells 107 to excitation light wave radiation of preprogrammed wavelengths and measures the fluorescence of the samples at preprogrammed emission wavelengths. The fluorescence of the solution in a given well can be measured a number of times and digital values representing each measurement are generated and stored. The excitation light source is a Xenon lamp 109 which generates electromagnetic radiation in the range of 300 to 1,000 nanometers wavelength. Light from lamp 109 is collected by a lens and fiber optic arrangement 111 and conveyed to an excitation fiber optic cable 117 by a software controllable optical filter 113. An optical exciter/sampler 119 which is attached to and extends through a relatively stationary mounting surface 123 (FIG. 3) of assay apparatus 98 receives the light conveyed by optical fiber 117. The light received by optical exciter/sampler 119 is distributed by a lens 121 to a fixed point of approximately the same area as well 107 beneath mounting surface 123 (FIG. 4) In the present example, the point beneath mounting plate 123 which receives light from lens 121 is called the sample point.

Lens 121 also collects light emitted from a well at the sample point beneath mounting surface 123 and couples the light so collected to a fiber optic cable 125. A software-

controllable emission optical filter 127 couples a selected range of wavelengths of the light conveyed by fiber optic cable 125 to a photo-multiplier tube 131 via a fiber optic cable 129. The magnitude of the light conveyed by fiber 129 is converted to an analog electrical signal from which a digital light amplitude representation is periodically generated.

The liquid reagent which is added to wells 107 is provided to assay apparatus 98 in pre-programmed quantities via tube 99. An outlet tip 101 of tube 99 is attached to and extends through mounting surface 123 at a fixed location relative to the attachment of exciter/sampler 119. In the present embodiment, fluid tip 101 and optical exciter/sampler 119 are mounted to surface 123 such that when a well e.g., 107c in a given row, is under the exciter/sampler 119, a well, e.g., 107b, two columns to the right (FIG. 2) in the same row is under fluid tip 101.

The multi-well plate 105 is carried in assay apparatus 98 by a plate-carrier system 133 which moves plate 105 in a rectangular coordinate manner to place any of the wells 107 under the exciter/sampler 119 or under the fluid tip 101 responsive to plate movement commands from microcomputer 90 (FIG. 3). Assay apparatus 98 includes an assay controller 134 which is connected to microcomputer 90 by bi-directional bus 102 (FIG. 4). Assay controller 134 operates in a manner disclosed in the Fluoroskan II descriptive material to receive commands from microprocessor 90 and implement functions specified in those commands. Assay controller 134 receives filter set up commands from microcomputer 90 to which it responds by rotating optical filters 113 and 127 to values specified in the filter set up commands. Also, assay controller 134 receives plate movement commands specifying a particular well 107 to move to a position under exciter/sampler 119 or reagent-adding tip 101. Assay controller 134 responds to such plate movement commands by controlling the plate movement system 133 to appropriately position the specified well. In addition, assay controller 134 generates digital representations of fluorescence magnitudes sensed by photo-multiplier tube 131 and returns those digital representations to microcomputer 90.

Automatic pipette 96 of the present embodiment is a modified version of the Digiflex-TP model 33020 manufactured by ICN Biomedicals, Inc. of Horsham, Pa. As is well known in the art and described in the Digiflex-TP operating manual, the automatic pipette 96 is capable of operating under microcomputer control to dispense precise amounts of selected fluids through an outgoing tube, such as tube 99. The Digiflex-TP includes a pair of syringes for dispensing fluids; however, in the present embodiment, only a single syringe 135 (FIG. 5) is used.

Automatic pipette 96 includes a pipette controller 139 which is connected to microcomputer 90 by bus 103 for controlling the functions of the automatic pipette. The specific means for exercising such control are well known from the Digiflex-TP operating manual and are not described in detail herein.

FIG. 5 shows selected components of automatic pipette 96 which are used in the present embodiment to dispense reagent to assay apparatus 98 via tube 99. Syringe 135 of the automatic pipette 96 includes a plunger 137 for drawing reagent into the syringe 135 and pumping the reagent from the syringe under the control of pipette controller 139. Syringe 135 also includes two-position valve 141 which, under control from pipette controller 139, selectively connects syringe 135 into fluid contact with outgoing tube 99 and with a reagent input tube 143. As previously described,

outgoing tube **99** conveys reagent to the wells **107** of multi-well plate **105** in the assay apparatus **98** via a fluid tip **101**. Reagent input tubing **143** conveys reagent from a reagent vessel **145** to the syringe **135**.

Pipette controller **139** receives set up commands from microcomputer **90** at the beginning of an assay operation. The set up commands pre-establish the amount of reagent to be drawn into syringe **135** responsive to a draw command and the amount of reagent to be pumped from syringe **135** responsive to a pump command. The set up commands also specify the rate at which reagent is to be drawn into the syringe **135** and pumped from the syringe. Three rates W1, W2 and W3, which represent fast, half-fast and one-fourth fast plunger speeds respectively, are available. In the present embodiment W1 is used.

Precisely controlled amounts of reagent can be delivered to assay apparatus **98** by a sequence of valve control and plunger control (draw and pump) commands after pre-establishing the amounts and rates of reagent to be moved by syringe **135**. First, a fluid-in command is sent to automatic pipette **96** to connect input tube **143** into fluid communication with syringe **135** via valve **141**. A draw command is then sent to automatic pipette **96** to which pipette controller **139** responds by drawing plunger **137** the pre-established amount at the pre-established rate. Microcomputer **90** then transmits a fluid output command to which pipette controller **139** responds by changing the position of valve **141** to connect syringe **135** to output tube **99**. When a pump command is received by pipette controller **139**, the plunger **137** is pushed into syringe **135** by the pre-established amount at the pre-established rate. Such plunger movement pumps the pre-established amount of reagent from syringe **135** and out of tip **101** into a well **107** of multi-well plate **105**.

FIG. 6 is a flow diagram of the functions performed by microcomputer **90** to coordinate an assay of a plurality of prepared solutions which have been placed in wells **107** of a multi-well plate **105**. Initially, the power is supplied to the apparatus of FIG. 1 and a prepared multi-well plate **105** is placed in the plate carrier system **133** (FIG. 2) of assay apparatus **98**. Upon power up, microcomputer **90** performs in a block **151** a start-up routine in which communication is established with both automatic pipette **96** and assay apparatus **98** over buses **103** and **102**, respectively. One important communication attribute, which is established during communication set-up, is the data rate (band rate) for signalling between the various units. After communication set-up, microcomputer **90** proceeds to a block **153** in which the parameter values for a test are established. In the present embodiment, a test consists of the assay of one or more wells using substantially the same test parameter values. At the end of a test, a new test of the same or other wells **107** may be established using different parameter values defining a new test.

To establish test parameter values in block **153**, the microcomputer **90** causes a list of parameter names to be displayed on monitor **92**. Such a list is shown in FIG. 7. A human operator by interaction with keyboard **91**, assigns a value to each of the displayed parameters, as is also shown in FIG. 7. The human interaction consists of the movement of a monitor cursor from one parameter name to the next, and the entry of a value for each of the parameters. After the test parameter values have been established, the flow proceeds to block **155** where the automatic pipette **96** is set-up to conduct the defined test. In block **155**, the reagent quantity draw of 50 microliters, the reagent quantity pump of 50 microliters, and the syringe speed of W2 are transmitted to

automatic pipette **96** via bus **103** (FIG. 1). The pipette controller **139** stores the set-up values for use in implementing draw and pump commands (FIG. 5).

A block **157** is next performed to prepare the assay apparatus **98** for the defined test. In block **157**, microcomputer **90** transmits to assay apparatus **98** via bus **102**, the values specified for both the excitation filter **113** and the emission filter **127**. The range of possible excitation and emission filter values are shown in Tables 1 and 2, respectively. In response to the filter values, the assay controller **134** adjusts the rotatable excitation filter **113** and the rotatable emission filter **127** to the requested filter settings. The remainder of the parameter values are kept within microcomputer **90** for use in controlling the defined test.

TABLE 1

Name	Excitation Filters		Half Power
	Wavelength	Efficiency	Band Width
ex1	355 nm	30%	35 ± 4 nm
ex2	485 nm	50%	14 ± 2 nm
ex3	544 nm	60%	15 ± 2 nm
ex4	584 nm	50%	16 ± 2 nm

TABLE 2

Name	Emission Filters		Half Power
	Wavelength	Efficiency	Band Width
em1	460 nm	60%	25 ± 3 nm
em2	538 nm	65%	25 ± 3 nm
em3	590 nm	30%	14 ± 2 nm
em4	612 nm	40%	6 ± 1 nm

The assay of the sample in a specified well includes the measurement of fluorescence of the sample in the specified well prior to adding reagent, the adding of reagent to the well and the measurement of fluorescence of the sample in the well after the addition of reagent. Upon completion of the assay of a well, the process repeats on other wells up to the number assigned to the number of wells parameter in block **153**.

The testing of the solution in the well identified as the start well begins in block **159** in which microcomputer **90** transmits to assay apparatus **98** a plate move command specifying the X, Y coordinate address of the test well which was set equal to the start well (3,3) in a block **158**. Assay controller **134** responds to the plate move command by controlling plate moving system **133** to move plate **105** to a position in which the test well (3,3) is at the sample point under the lens **121** of optical exciter/sampler **119**. The excitation optical radiation from fiber **117** then excites the solution in the test well (3,3). A measure command is then transmitted in step **161** to assay apparatus **98** to measure the fluorescence of the solution in the test well. Responsive to the measure command, assay controller **134** generates a digital representation of the fluorescence magnitude sensed by photo-multiplier **131** which representation is transmitted to microcomputer **90** for storage therein in block **163**. A loop comprising blocks **161**, **163**, **165** and **166** is performed the number of times specified in FIG. 7 for the parameter pre-reagent reads. The block **166** delays the passage through the loop so that successive measure commands are transmitted by block **161** at approximately 635 millisecond intervals.

When decision block **165** determines that the pre-reagent measurements are complete, flow proceeds to a block **167**

where automatic pipette **96** is sent a fluid in command and a draw command. In response to the fluid in command, pipette controller **139** moves valve **141** to connect syringe **135** to tube **143** and in response to the draw command, 50 microliters of reagent are drawn into syringe **135**. Substantially simultaneously with the transmission of the draw command, the microcomputer **90** begins the movement of the pre-reagent fluorescence values recorded in step **163** to its associated disk store **94** in block **169**. The pre-reagent values are stored in a file "Test 1" as specified in the input parameters (FIG. 7), at a location allocated to pre-reagent measurements of well **3,3**. Next, a block **171** is performed in which a plate move command specifying that the test well (**3,3**) be moved to the reagent-adding position under fluid tip **101**. Following the move command of block **171** by a period of time in which the movement will be completed, a fluid-out command and a pump command are transmitted in block **173** by microcomputer **90** to automatic pipette **96**. Responsive to the fluid-out and pump commands, valve **141** is switched and 50 microliters of reagent are pumped into tube **99**, thus forcing 50 microliters of reagent out of fluid tip **101** into the test well. After a brief pause to allow completion of the pump operation, a step **175** is performed in which assay apparatus **98** is commanded to move the test well to the measure position under exciter/sampler **119**. Advantageously, the test well is at the measure point within approximately 2 seconds of receiving the reagent.

Microcomputer **90**, after a brief pause to permit the completion of plate movement, begins a loop consisting of blocks **177**, **179**, **181** and **182** in which **125** fluorescence measurements are taken from the test well as specified in the post-reagent input value of FIG. 7. During each trip through the loop a sample command is transmitted to assay apparatus **98** which responds thereto by returning to microcomputer **90** a digital representation of fluorescence. All returned digital representations are stored in step **179**. Block **182** delays the passage through the loop so that successive measure commands are transmitted in block **177** at approximately 635 millisecond intervals. Decision block **181** counts the number of received digital fluorescent representations until the specified number of 125 such representations have been received and stored. After the 125th digital fluorescence value has been received from assay apparatus **98**, the flow proceeds to block **183** where the 125 post-reagent fluorescence representations are transferred to disk store **94**.

Next, a comparison block **185** is performed to determine if all of the wells specified in the test parameters have been measured. In the present example, eight wells are to be measured as indicated by the test input parameters (FIG. 7). Accordingly, flow proceeds back to block **159** after the address of the test well is set in a block **186** to the address of the next well (**3,4**) to be measured. In the present embodiment, the next well is the immediately adjacent well down (larger Y address) the present column of wells. When the last well of a column, e.g., **3,8** has been measured the next well is the first well of the immediately succeeding column, e.g., **4,1**. After returning to block **159**, tests continue for each of the remaining wells in accordance with the previously described blocks **159** through **185**. After the last well has been measured, block **185** directs program flow to a block **187** in which a question is displayed on monitor **92** asking if another test is to be performed. When another test is to be performed, flow returns from block **187** to block **153** in which a subsequent test may be defined and performed as previously described. If no new test is specified by an operator, flow proceeds from decision block **187** to decision block **189**. In block **189**, microcomputer **90** asks via the

monitor **92** whether reports are to be generated. The operator can indicate that no reports are needed at this time, and flow proceeds to a block **191** in which the assay program ends. Alternatively, when the operator indicates in block **189** that reports are to be generated a report sequence (not shown) is entered in which data accumulated from the tests performed can be reported.

FIG. 8 represents one report which can be generated in Step **193** by the apparatus of FIG. 1. The report of FIG. 8 is generated by reading the fluorescence of each well and plotting fluorescence as a function of time. The relative time of each measurement is easily determined since successive measurements for a well are taken at the previously discussed 635 millisecond intervals.

As previously described, assay apparatus **98** comprises a modified and improved Fluoroskan II. One additional modification of the Fluoroskan II which improves the consistency of test results, is the placement of a surface having consistent non-reflective properties between the multi-well plate **105** and the plate carrier system **133**. In the preferred embodiment, a sheet of heavy black paper was cut to fit the plate carrier system **133** and placed between the plate carrier system and the multi-well plate **105** before measurements were made.

While the apparatus of the present invention has been described with reference to a movable plate-carrying component for moving, in a controlled step-wise sequence, a predetermined well into alignment with a stationary reagent-delivering outlet and a stationary fluorescence measurement device, it will be understood that the apparatus could be configured so that the plate carrier remains stationary and the reagent-delivering outlet and the fluorescence-measurement device move, together or independent of one another, into alignment with a predetermined well.

In accordance with another of its aspects the present invention entails an automated method for detecting activity of cell surface ion channels and/or receptors of a cell, wherein the ion channel or receptor, when activated, directly or indirectly contributes to a detectable change in the cytoplasmic level of a predetermined ion in the cell, the cytoplasm of which cell contains an indicator which is sensitive to said ion, and wherein the method is carried out in an apparatus which is capable of (1) delivering a reagent solution to a predetermined cell-containing compartment of a vessel and (2) measuring the detectable change in the cytoplasmic level of the ion in the cells of said predetermined compartment, the method comprising the steps of:

- (a) providing a divided culture vessel having an array of individual compartments at least one of which contains viable cells comprising functional ion channels and/or receptors which, when activated, are capable of directly or indirectly causing a detectable increase in the concentration of the predetermined ion in the cytoplasm, the cytoplasm of said cells comprising an amount of an ion-sensitive fluorescent indicator sufficient to detect a change, if any, in the concentration of said predetermined ion;
- (b) aligning a predetermined cell-containing compartment with a fluid outlet of an automatic pipette and delivering an aliquot of a solution comprising an amount of a known or putative ion channel- or receptor-activating compound which is effective to activate said ion channel or receptor; and
- (c) aligning said cell-containing compartment with a fluorescence detection means and detecting and measuring for a predetermined amount of time fluorescence

emitted by the ion-sensitive indicator in response to an excitation wavelength.

In accordance with the various assays of the present invention, cells are employed which have ion channels or receptors, the activation of which results in an increase in the level of a cation or anion in the cytoplasm. The cytoplasm of the cells employed are loaded with a fluorescent indicator which is sufficiently sensitive to said ion. By the phrase “sufficiently sensitive fluorescent indicator” is meant a fluorescent compound which, in the presence of, and over a range of physiological concentrations of, a particular ion, is capable of producing distinguishable levels of fluorescence intensity. Preferably, a fluorescent indicator should be able to produce detectably different intensities of fluorescence in response to relatively small changes in ion concentration and produce an intensity of fluorescence at peak cytoplasmic levels (after activation) which is at least about 50% higher, more preferably at least 100–200% higher than the fluorescence intensity in the cytoplasm of the cell when it is at rest.

One embodiment of the assays of the present invention for determining ion channel or receptor activity and compounds that affect such activity, concerns the use of “direct” assays. As used herein direct assays describe assays employing cells loaded with a fluorescent indicator which is capable of binding a specific ion, which cells have ion channels or receptors that are permeable, when activated, to said ions. Such direct assays may be performed to assay, for example, cells loaded with a calcium-sensitive fluorescent indicator and having receptors and/or ion channels that flux calcium (e.g., calcium channels or NMDA receptors, cells loaded with a chloride-sensitive fluorescent indicator and having receptors which are permeable to chloride ions (e.g., GABA receptors), cells loaded with a sodium- or potassium-sensitive fluorescent indicator and having receptors which are permeable to sodium or potassium ions (e.g., kainate/AMPA receptors and nicotinic acetylcholine receptors), and so forth.

In especially preferred embodiments of the present invention cells are used which have voltage dependent calcium channels and are loaded with a calcium-sensitive fluorescent indicator. Calcium ions may enter the cytoplasm from the extracellular medium (where the concentration of calcium ions is greater) when voltage dependent calcium channels are activated by depolarization of the cell membrane. Various embodiments of the assays of the present invention use cells which express functional calcium channels. In assays directed to determining the activity of calcium channels, the calcium channels are activated by addition of potassium ions to the extracellular medium which depolarizes the cell membrane.

Moreover, it is also known that certain ligand-gated ion channels, such as the GluR1 or GluR3 subtypes of EAA receptors and nicotinic acetylcholine receptors, can flux calcium ions, particularly in sodium-free medium. Assays for determining activity of such receptors, employing cells devoid of functional calcium channels (or using calcium channel-specific blockers) can be used such that receptor activity may be measured by increased levels of calcium in the cytoplasm. As those of ordinary skill in the art will understand, sodium-free medium may be supplemented with an appropriate concentration of a composition for osmotic support (e.g., sodium-free Ringer solution supplemented with N-methyl D-glucamine, choline chloride or the like).

In accordance with other embodiments of the assays of the invention, the activity of receptors which are ligand-gated ion channels is determined in a type of “indirect” assay which utilizes a characteristic depolarization caused by the

flux of ions through ligand-gated ion channels. Such indirect assays employ cells having voltage-dependent calcium channels and the ligand-gated ion channels of interest. Activation of the ligand-gated ion channel causes ions (not calcium ions) to flux, depolarizing the cell membrane which in turn activates voltage-dependent calcium channels and results in the flow of calcium ions into the cytoplasm. The cytoplasm of the cells is loaded with a calcium-sensitive indicator. For example, activation of the nicotinic acetylcholine receptors by nicotine results in flux of sodium ions, depolarizing the cell membrane and, consequently, activating voltage dependent calcium channels. The degree of activation of the nicotinic receptors is measured indirectly by the flux of calcium ion through activated calcium channels. Among the known ligand-gated ion channels that could be assayed in this manner are receptors of the kainate/AMPA type including excitatory amino acid (EAA) receptors (e.g., glutamate and aspartate).

Still further embodiments of the assays of the present invention concern determining the activity of receptors the activation of which initiates subsequent intracellular events in which intracellular stores of calcium ions are released for use as a second messenger. Activation of some G-protein-coupled receptors stimulates the formation of inositol triphosphate (IP<sub>3</sub>, a G-protein-coupled receptor second messenger) through phospholipase C-mediated hydrolysis of phosphatidylinositol, Berridge and Irvine (1984). *Nature* 312: 315–21. IP<sub>3</sub> in turn stimulates the release of intracellular calcium ion stores. Thus, in accordance with the certain assays of the present invention, a change in cytoplasmic calcium ion levels caused by release of calcium ions from intracellular stores is used to reliably determine G-protein-coupled receptor function. This is another type of indirect assay. Among G-protein-coupled receptors are muscarinic acetylcholine receptors (mAChR), adrenergic receptors, vasoactive intestinal (VIP) peptide receptors, serotonin receptors, dopamine receptors, angiotensin receptors, adenosine receptors, bradykinin receptors, metabotropic excitatory amino acid receptors and the like. Cells expressing such G-protein-coupled receptors may exhibit increased cytoplasmic calcium levels as a result of contribution from both intracellular stores and via activation of ion channels, in which case it may be desirable although not necessary to conduct such assays in calcium-free buffer, optionally supplemented with a chelating agent such as EGTA, to distinguish fluorescence response resulting from calcium release from internal stores.

Any cell expressing a cell surface protein which is capable, upon activation, of directly increasing the intracellular concentration of calcium such as by opening gated calcium channels or indirectly affecting the concentration of intracellular calcium as by causing initiation of a reaction which utilizes Ca<sup>2+</sup> as a second messenger (e.g., G-protein-coupled receptors), may be used in the assay. Cells endogenously expressing such receptors or ion channels and cells which may be transfected with a suitable vector encoding one or more such cell surface proteins are known to those of skill in the art or may be identified by those of skill in the art. Although essentially any cell which expresses endogenous ion channel and/or receptor activity may be used, it is preferred to use cells transformed or transfected with heterologous DNAs encoding such ion channels and/or receptors so as to express predominantly a single type of ion channel or receptor. Many cells may be genetically engineered to express a heterologous cell surface protein are known. Such cells include, but are not limited to, Ltk cells, human embryonic kidney (HEK) cells, Chinese hamster

ovary (CHO) cells, PC12 cells and COS-7 cells. Exemplary cell surface proteins include, but are not limited to, cell surface receptors and ion channels.

Cell surface receptors include, but are not limited to, muscarinic receptors (e.g., human K2 (GenBank accession #M16404); rat M3 (GenBank accession #M16407); human M4 (GenBank accession #M16405); human M5 (Bonner, et al., (1988) *Neuron* 1, pp. 403–410); and the like; neuronal nicotinic acetylcholine receptors (e.g., the  $\alpha_2$ ,  $\alpha_3$  and  $\beta_2$  subtypes disclosed in U.S. Ser. No 504,455 (filed Apr. 3, 1990), hereby expressly incorporated by reference herein in its entirety); the rat  $\alpha_2$  subunit (Wada, et al. (1988) *Science* 240, pp. 330–334); the rat  $\alpha_3$  subunit (Boulter, et al. (1986) *Nature* 319, pp. 368–374); the rat  $\alpha_4$  subunit (Goldman, et al. (1987) *Cell* 48, pp. 965–973); the rat  $\alpha_5$  subunit (Boulter, et al. (1990) *J. Biol. Chem.* 265, pp. 4472–4482); the rat  $\beta_2$  subunit (Deneris, et al. (1988) *Neuron* 1, pp. 45–54) the rat  $\beta_3$  subunit (Deneris, et al. (1989) *J. Biol. Chem.* 264, pp. 6268–6272); the rat  $\beta_4$  subunit (Duvoisin, et al. (1989) *Neuron* 3, pp. 487–496); combinations of the rat  $\alpha$  subunits,  $\beta$  subunits and  $\alpha$  and  $\beta$  subunits; GABA receptors (e.g., the bovine  $\alpha_1$  and  $\beta_1$  subunits (Schofield, et al. (1987) *Nature* 328, pp. 221–227); the bovine  $\alpha_2$  and  $\alpha_3$  subunits (Levitan, et al. (1988) *Nature* 335, pp. 76–79); the  $\gamma$ -subunit (Pritchett, et al. (1989) *Nature* 338, pp. 582–585); the  $\beta_2$  and  $\beta_3$  subunits (Ymer, et al. (1989) *EMBO J.* 8, pp. 1665–1670); the  $\delta$  subunit (Shivers, B. D. (1989) *Neuron* 3, pp. 327–337); and the like); glutamate receptors (e.g., receptor isolated from rat brain (Hollman, et al. (1989) *Nature* 343, pp. 643–648); and the like); adrenergic receptors (e.g., human  $\beta_1$  (Frielle, et al. (1987) *Proc. Natl. Acad. Sci.* 84, pp. 7920–7924); human  $\alpha_2$  (Kobilka, et al. (1987) *Science* 238, pp. 650–656); hamster  $\beta_2$  (Dixon, et al. (1986) *Nature* 321, pp. 75–79); and the like); dopamine receptors (e.g., human D2 (Stormann, et al. (1990) *Molec. Pharm.* 37, pp. 1–6); rat (Bunzow, et al. (1988) *Nature* 336, pp. 783–787); and the like); NGF receptors (e.g., human NGF receptors (Johnson, et al. (1986) *Cell* 47, pp. 545–554); and the like); serotonin receptors (e.g., human 5HT1a (Kobilka, et al. (1987) *Nature* 329, pp. 75–79); rat 5HT2 (Julius, et al. (1990) *PNAS* 87, pp. 928–932); rat 5HT1c (Julius, et al. (1988) *Science* 241, pp. 558–564); and the like).

Ion channels include, but are not limited to, calcium channels comprised of the human calcium channel,  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$  and/or  $\gamma$ -subunits disclosed in commonly owned U.S. application Ser. No. 745,206, filed Aug. 15, 1991, now U.S. Pat. No. 5,429,921, the contents of which are hereby incorporated by reference; (see also, W089/09834; human neuronal  $\alpha_2$  subunit); rabbit skeletal muscle  $\alpha_1$  subunit (Tanabe, et al. (1987) *Nature* 328, pp. 313–E318); rabbit skeletal muscle  $\alpha_2$  subunit (Ellis, et al. (1988) *Science* 241, pp. 1661–1664); rabbit skeletal muscle  $\beta$  subunit (Ruth, et al. (1989) *Science* 245, pp. 1115–1118); rabbit skeletal muscle  $\gamma$  subunit (Jay, et al. (1990) *Science* 248, pp. 490–492); and the like); potassium ion channels (e.g., rat brain (BK2) (McKinnon, D. (1989) *J. Biol. Chem.* 264, pp. 9230–9236); mouse brain (BK1) (Tempel, et al. (1988) *Nature* 332, pp. 837–839); and the like); sodium ion channels (e.g., rat brain I and II (Noda, et al. (1986) *Nature* 320, pp. 188–192); rat brain III (Kayano, et al. (1988) *FEBS Lett.* 228, pp. 187–194); and others).

The preparation of cells which express a receptor cell surface protein capable of increasing intracellular  $\text{Ca}^{2+}$  level in response to ligand binding membrane or depolarization which cells are useful for testing compounds to assess the effects of ligand-receptor interaction is exemplified in the Examples provided herewith by reference to mammalian Ltk

cells which have been transfected so as to express the Type 1 human muscarinic (HM1) receptor or HEK 293 cells which have been transfected so as to express human neuronal calcium channel subunits. SHSY5Y or IMR32 cells which express endogenous human muscarinic receptor, nicotinic receptor or calcium channel proteins also can be useful for testing compounds in the present assay. The assays of the present invention may also be used to detect compounds which affect functional growth factor receptors, such as nerve growth factor (NGF), heparin binding growth factors and other growth factors.

Activation of the cellular receptors and/or ion channels of interest in accordance with the present invention may result in a transient increase in the level of intracellular calcium. The initial increase in calcium may be detected as an increase in fluorescence within as soon as one to two seconds after the addition of the reagent which activates the receptors and/or ion channels and is usually short-lived. Fluorescence levels in the cytoplasm typically increase to a peak value and then typically decline as excess calcium ions are removed by normal cellular mechanisms. Typically, receptor or ion channel activation causes fluorescence levels to peak within about 5 to about 45 seconds followed by reduction in fluorescence for about 2 to 20 minutes until intracellular calcium levels approach pre-activation levels. The speed at which the fluorescence can be analyzed is very important due to the kinetics of the response reaction contributed by an increase in calcium ions in the cytoplasm followed by a subsequent decrease in the level of calcium ions as they are removed from the cytoplasm.

The cells used in the assays of the invention are loaded with a fluorescent indicator which is sufficiently sensitive as to produce detectable changes in fluorescence intensity in response to changes in the concentration of the ions in the cytoplasm. It is particularly preferred to use a fluorescent indicator which has such sensitivity in the presence of calcium ions, although indicators sensitive to other ions such as sodium ions, potassium ions, chloride ions, and the like may be employed depending on the type of receptor or ion channel activity being studied, as will be understood by the person of ordinary skill in the art. Thus, in especially preferred embodiments of the automated assays for determining functional activity of cell surface proteins and identifying compounds which have the capacity to modulate cell surface protein function (i.e., agonists, antagonists, potentiators, etc.) changes in the level of ions in the cytoplasm are detected by changes in fluorescence of a ion-sensitive indicator. Among the ion-sensitive indicators which may be employed are those disclosed in the following Table (Table 3).

TABLE 3

ION SENSITIVE FLUORESCENT DYES	
Fluorescent Indicator Name	Molecular Probes, Inc. Eugene, OR 97402 Catalog #
Calcium-sensitive	
Quin-2,AM	Q-1288
Fura-2,AM	F-1225
Indo-1,AM	I-1226
Fura-3,AM	F-1228
Fluo-3,AM	F-1241
Rhod-2,AM	R-1244
BAPTA,AM	B-1205
5,5'-dimethyl BAPTA,AM	D-1207



TABLE 3-continued

ION SENSITIVE FLUORESCENT DYES	
Fluorescent Indicator Name	Molecular Probes, Inc. Eugene, OR 97402 Catalog #
4,4'-difluoro BAPTA,AM	D-1216
5,5'-difluoro BAPTA,AM	D-1209
5,5'-dibromo BAPTA,AM	D-1213
Calcium Green	C-3011
Calcium Orange	C-3014
Calcium Crimson	C-3017
Fura-5	F-3023
Fura-Red	F-3020
<u>Sodium-sensitive</u>	
SBFI	S-1262
<u>Potassium-sensitive</u>	
PBFI	P-1265
<u>Magnesium-sensitive</u>	
Mag-Fura-2,AM	M-1291
Mag-Indo-1,AM	M-1294
Mag-Quin-2,AM	M-1299
Mag-Quin-1,AM	M-1297
<u>Chloride-sensitive</u>	
SPQ	M-440
SPA	S-460

Particularly preferred assays of the present invention employ a calcium-specific fluorescent indicator, such as fluo-3, fura-2, calcium green, or the like. The most preferred calcium-specific indicator is fluo-3. See, A. Minta, et al., *J. Biol. Chem.* 264:8171–8178 (1989); J. P. Y. Kao, et al., *J. Biol. Chem.* 264:8179–8184 (1989). Other fluorescent indicators for detecting ion concentrations and other fluorescent compounds such as amino acid-fluorescent indicator conjugates can also be used to detect the activity of a cell surface protein or the effect of a compound on cellular function wherein the fluorescent compound has differential fluorescing intensities in the presence and absence of the cellular function of interest. The generally hydrophilic fluorescent indicators may be loaded into the cytoplasm by incubating the cells with a solution comprising a membrane-permeable derivative of the dye; however the process may be facilitated where a more hydrophobic form of the indicator is used. Thus, fluorescent indicators are known and available as more hydrophobic acetoxymethyl esters (AME) which are able to permeate cell membranes much more readily than the unmodified dyes. When the acetoxymethyl ester form of the dye enters the cell, the ester group is removed by cytosolic esterases, thereby trapping the dye in the cytosol. Fluorescent indicator-loading may be carried out at a temperature between about 5° C. and about 37° C. for between about 0.25 hours and about 20 hours, using a concentration of indicator in the range of about 5  $\mu$ M to about 100  $\mu$ M. Loading at 25° C. for 2–3 hours gives good results. Once the cells have been loaded, they are washed with e.g., a buffered saline solution. After the cells are washed, they should be used in an assay within about 20 minutes.

In accordance with especially preferred embodiments of the present invention the assay reactions are carried out in a microtiter plate having a plurality of compartments (i.e., wells) seeded with viable cells which express voltage-dependent calcium channels and/or receptors of interest. Plastic microtiter plates having 96 wells arranged in a 12-by-8 array, as well as other arrays (e.g., 6-by-4 or 4-by-3), are well known in the art. Although any number of

cells capable of eliciting a detectable fluorescence signal in an assay may be used, the number of cells seeded into each well may desirably be chosen so that the cells are at or near confluence, but not overgrown, when the assays are conducted, so that the fluorescence signal-to-noise ratio is increased.

After the cells have been loaded with the fluorescent dye, the cells in the microtiter plate are washed and suitable assay buffer is added to the respective wells. If desired, the assays may be carried out in replicate wells (duplicate, triplicate, etc.) for statistical purposes. The buffer conditions in the individual wells should be such that the ion channels or receptors of interest in the assay will remain substantially at rest (i.e., not activated) until stimulated by addition of an aliquot of reagent which activates the ion channel or receptor of interest. The microtiter plate is placed in the apparatus of the invention just prior to starting a series of individual assays in the respective wells. The assays may be carried out at a temperature of about 10° C. to about 37° C., more preferably at about 20° C. to about 30° C.

Prior to starting the sequence of multiple assays, certain variables may be keyed into a controlling computer such as a microprocessor. As described in detail hereinbefore, variables may include the number and location (i.e., column, row) of the first well to be assayed, the number of data points to be collected, the time period for obtaining and recording fluorescence readings (background and post-activation), the filter settings for excitation and emission wavelengths, the aliquot size to be delivered by the automatic pipetter, and the like.

The apparatus of the present invention coordinates the movement of the microtiter plate, reading of the fluorescence signal, and initiation of the assay reaction by addition of an aliquot of reagent to an individual well to activate the ion channels or receptors of interest. In preferred embodiments, the first well to be tested is aligned with optical exciter/sampler 119 and background fluorescence emission from the cells is measured for a short period of time. Then the well is aligned with the fluid tip 101 of automatic pipette 96 where an aliquot of a suitably concentrated reagent is added so as to initiate the assay reaction, and then realigned with the optical exciter/sampler.

Any compound which is known to activate ion channels or receptors of interest may be used to initiate the assay. Choosing an appropriate ion channel- or receptor-activating reagent depending on the ion channel or receptor of interest is within the skill of the art. Direct depolarization of the cell membrane to determine calcium channel activity may be accomplished by adding a potassium salt solution having a concentration of potassium ions such that the final concentration of potassium ions in the cell-containing well is in the range of about 50–150 mM (e.g., 50 mM KCl). With respect to ligand-gated receptors and ligand-gated ion channels, ligands are known which have affinity for and activate such receptors. For example, nicotinic acetylcholine receptors are known to be activated by nicotine or acetylcholine; similarly, muscarinic acetylcholine receptors may be activated by addition of muscarine or carbamylcholine. Where it is desirable to conduct assays concerning compounds having unknown or putative ligand activity, the assays may be initiated by delivering a reagent comprising the compound through fluid tip 101 of automatic pipette 96.

Agonist assays may be carried out on cells known to possess ion channel or receptor to determine what affect, if any, a compound has on activation or potentiation of ion channels or receptors of interest. Agonist assays also may be carried out using a reagent known to possess ion channel- or

receptor- activating capacity to determine whether a cell expresses the respective functional ion channel or receptor of interest.

Contacting a functional receptor or ion channel with agonist activates a transient reaction; and prolonged exposure to agonist may desensitize the receptor or ion channel to subsequent activation. Thus, in general, the assays for determining ion channel or receptor function should be initiated with addition of agonist (i.e. in the reagent solution used to initiate the reaction). The potency of a compound having agonist activity is determined by the detected increase in fluorescence in the cells as compared to the level of fluorescence in either the same cell, or substantially identical cell, which is treated substantially identically except that reagent lacking the agonist (i.e., control) is added to initiate the reaction. Where an agonist assay is performed to test whether or not a cell expresses the functional receptor or ion channel of interest, using known agonist, in addition to test-cell-containing wells, known agonist is added to wells containing control cells (substantially identical cell that lacks the specific receptors or ion channels) and the levels of fluorescence are compared. Depending on the assay, cells lacking the ion channel and/or receptor of interest should exhibit substantially no increase in fluorescence in response to the known agonist. A substantially identical cell may be derived from the same cells from which recombinant cells are prepared but which have not been modified by introduction of heterologous DNA. Alternatively, it may be a cell in which the specific receptors or ion channels are removed. Any statistically or otherwise significant difference in the level of fluorescence indicates that the test compound has in some manner altered the activity of the specific receptor or ion channel or that the test cell possesses the specific functional receptor or ion channel. In especially preferred embodiments of the present invention which pertain to automated drug screening assays for identifying compounds which have the ability to activate ion channels or receptors of interest, it is contemplated that each of the individual wells (or duplicate wells, etc.) contain a distinct cell type, or distinct recombinant cell line expressing a homogenous population of a receptor or ion channel of interest, so that the compound having unidentified activity may be screened to determine whether it possesses agonist activity with respect to one or more of a variety of functional ion channels or receptors.

In another of its aspects the invention entails automated antagonist assays. Antagonist assays, including drug screening assays, may be carried out by incubating the cells having functional ion channels and/or receptors in the presence and absence of one or more compounds, added to the solution bathing the cells in the respective wells of the microtiter plate for an amount of time sufficient (to the extent that the compound has affinity for the ion channel and/or receptor of interest) for the compound(s) to bind to the receptors and/or ion channels, then activating the ion channels or receptors by addition of known agonist, and measuring the level of fluorescence in the cells as compared to the level of fluorescence in either the same cell, or substantially identical cell, in the absence of the putative antagonist.

As will be understood by the person of ordinary skill in the art, compounds exhibiting agonist or antagonist activity in an ion channel or receptor assay based on changes in intracellular ion levels will result in stimulation or enhancement (agonist) or inhibition (antagonist) of an increase in the intracellular concentration of calcium ions after activation of the ion channels or receptors of interest. It is desirable to measure the amount of agonist or antagonist activity in a

linear range of the assay system, such that small but significant increases or decreases in fluorescence relative to control well (e.g., devoid of the test compound) may be observed. It is well within the skill of the art to determine a size and concentration of a reagent solution which causes a suitable activation response in cells so that potentiation and/or inhibition of the ion channels or receptors of interest may be reliably detected.

With respect to voltage-dependent calcium channels, certain agonists are known (e.g., Bay K 8644) which act to increase the duration of time that calcium channels remain open after membrane depolarization but do not cause depolarization. Because such agonists bind to ion channels without activating them, they may be assayed in a manner similar to that used for an antagonist assay except that the observed result will be increased fluorescence in the test wells.

Immediately after addition of the reagent which initiates activation of the ion channels or receptors of interest, the plate is moved, if necessary, so that the cell-containing assay well is positioned for measurement of fluorescence emission. Because development of a fluorescence signal may begin within the first few seconds after addition of ligands or other reagents, it is desirable to move the assay well from the pipetting station to the fluorescence reading station as quickly as possible, times of about two seconds or less being desirable. The well should remain at the fluorescence reading station for a predetermined period of time suitable to measure and record the increase of intracellular calcium. In preferred embodiments of the invention the fluorescence after activation is read and recorded until fluorescence peaks and falls. An empirically determined time period may be chosen which covers the transient rise and fall of intracellular calcium levels in response to addition of the reagent. It has also been found, surprisingly, that placing a sheet of black paper beneath the microtiter plate in the Fluoroskan II (and presumably other fluorescent plate reading instruments) increases accuracy of measuring the fluorescent signal in this particular instrument.

After finishing reading and recording the fluorescence in one well, the just described apparatus steps are repeated with the next well in the series so as to measure background fluorescence, add reagent and measure and record the transient increase, if any, fluorescence. The apparatus of the present invention is programmable to begin the steps of an assay sequence in a predetermined first well and proceed sequentially down the columns and across the rows of the plate in a predetermined route through well number n.

The fluorescence data from replicate wells of cells treated in the same manner are collected and recorded (e.g., stored in the memory of a computer) for calculation of fluorescence signal-to-noise ratio (S/N) and/or the intracellular calcium signal-to-noise ratio. Fluorescence readings, corresponding to  $F_B, F_D, F_{max}, F_{min}$ , can be obtained from each drug-treated well. These values (as defined below) can be inserted into Equations 1 and 2 to calculate the signal-to-noise ratios of fluorescence (F) and of intracellular calcium ( $Ca_i$ ).

$$F S/N = \frac{F_D - F_{min}}{F_B - F_{min}} \quad \text{Equation 1}$$

$$Ca_i S/N = \frac{\frac{F_D - F_{min}}{F_{max} - F_D} \times 450 (K_d \text{ for } fluo-3)}{\frac{F_B - F_{min}}{F_{max} - F_B} \times 450} \quad \text{Equation 2}$$

$F_B$  = fluorescence reading before drug addition

$F_D$  = fluorescence reading after drug addition

$F_{max}$  = fluorescence reading after cell lysis with Triton

X-100

$F_{min}$  = fluorescence reading after quenching with EGTA.

As shown in Equation 1, the fluorescence signal-to-noise ratio represent the ratio of the fluorescence measured in the presence of the drug (signal) and the fluorescence measured in the presence of buffer only (noise). The background fluorescence,  $F_{min}$  (measured after EGTA quenching), was subtracted from both the signal fluorescence and the noise fluorescence. Alternatively, meaningful results can be obtained by simply calculating fluorescence signal-to-noise as the ratio of the peak fluorescence after addition of a compound and the total fluorescence before addition of a compound. The intracellular calcium signal-to-noise ratios represent the ratio of the intracellular calcium concentrations in the presence of the drug and in the presence of buffer only as calculated from the fluorescence measurements (see Equation 2).

The fluorescence dye-based assays of the present invention are thus useful for rapidly screening compounds to identify those that interact with any cell surface protein whose activity ultimately results in an altered concentration of ions in the cytoplasm of a cell. In particular, the assays can be used to test functional ligand-receptor or ligand-ion channel interactions for cell surface-localized receptors including ligand-gated ion channels, voltage-gated ion channels, G-protein-coupled receptors and growth factor receptors.

While the preferred assays of the present invention involve detecting differences in fluorescence intensities based on changes in the level of fluorescence intensity of an ion-sensitive fluorescent indicator caused by changes in the level of a particular ion in the cytoplasm, it will be understood that the assays of the present invention encompass measuring a detectable change of a solution as a consequence of a cellular event which allows a compound, which is capable of differential fluorescence to change its fluorescent intensity in response to the cellular event. By selecting a particular fluorescent compound which is capable of differential fluorescence intensities upon the occurrence of a cellular event, various assays which are within the scope of the present invention may be performed. For example, assays for determining the capacity of a compound to induce cell injury or cell death may be carried out by loading the cells with a pH sensitive fluorescent indicator such as BCECF (Molecular Probes, Inc., Eugene, Oreg. 97402, Catalog #B1150) and measuring cell injury or cell death as a function of changing fluorescence over time.

Furthermore, assays may be based on an increase in fluorescence when a fluorescent indicator is released from the cytoplasm of a cell. For example, assays for determining the ability of a chemotherapeutic agent to kill malignant cells may be carried out using malignant cells which are loaded with a fluorescent indicator which is sensitive to an ion (e.g.,  $Ca^{2+}$ ) wherein the ion is present at much greater

concentration in the solution bathing the cell than in the cytoplasm, such that cytotoxicity of the test compound is measured by the flow of dye out of the cell into the extracellular medium (i.e., from an environment of low calcium concentration to an environment of high calcium concentration) resulting in an increased intensity of fluorescence emission by the indicator.

Moreover, the differential intensity of fluorescence, which is detected by the assay methods of the present invention may be caused by a modification of a compound which can exist in either a non-fluorescing or fluorescing form and wherein occurrence of the cellular event results in a modification which allows the non-fluorescing form of the compound to become fluorescent. In an example of such an assay, the function of certain neurotransmitter transporters which are present at the synaptic cleft at the junction between two neurons, may be determined by the development of fluorescence in the cytoplasm of such neurons when conjugates of an amino acid and fluorescent indicator (wherein the fluorescent indicator of the conjugate is an acetoxymethyl ester derivative e.g., 5-(aminoacetamido) fluorescein; Molecular Probes, Catalog # A1363) and are transported by the neurotransmitter transporter into the cytoplasm of the cell where the ester group is cleaved by esterase activity and the conjugate becomes fluorescent.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

#### EXAMPLE 1

##### Preparation of Stably Transfected Mammalian Cell Lines That Express Human M1 *Muscarinic* Acetylcholine (HM1) Receptors

Stable cell lines expressing recombinant HM1 receptors for use in the automated assay were prepared. Ltk<sup>-</sup> cells, which are thymidine kinase-deficient mouse fibroblast cell lines, were stably co-transfected with a plasmid containing DNA that encodes EM1 and a selection plasmid containing either the wild-type or crippled thymidine kinase gene.

##### A. Preparation of transfected mammalian cell lines

The following cell lines can be used as host cells: HEK 293, which are human embryonic kidney cells available from ATCC (accession #CRL 1573); Ltk<sup>-</sup> cells, which are available from ATCC (accession #CCL1.3); COS-7 cells, which are available from ATCC (accession #CRL 1651); DG44 (see, e.g., L. Chasin (1986) Cell. Molec. Genet. 12, p. 555); and Chinese hamster ovary (CHO) cells.

B. DNA that encodes M1 receptor was cloned and inserted into a mammalian cell expression plasmid

The sequence of the HM1-encoding DNA fragment is described in Allard, et al. (1987), Nucl. Acids Res. 15, p. 10604. It can be prepared by synthesizing the cDNA construct based on the sequence described by Allard, et al. or it can be isolated by screening a human genomic DNA library. It has been isolated by screening a human genomic library that contains 2.5–4.5 kb-sized EcoRI fragments in the  $\lambda$ gt11 vector, with an oligonucleotide homologous to nucleotides 250–279 of the HM1 gene sequence. Screening conditions employed were as follows:

hybridization: 20% deionized formamide, 5×Denhardt's, 6×SSPE, 0.2% SDS, 200  $\mu$ g/ml sonicated herring sperm DNA, 42° C.

wash: 0.2×SSPE, 0.2% SDS, 50° C.

A positive clone was identified and confirmed to encode the HM1 receptor by DNA sequencing. The EcoRI insert of

that clone was isolated and inserted into the EcoRI site of pIB124 (International Biotechnologies, Inc., New Haven, Conn.), yielding clone pIBI24/HM1.

The HM1-encoding fragment of pIBI24/HM1 was modified for insertion into the SV40 promoter-based plasmid pSV2dhfr (see Subramani, et al. (1981) Mol. Cell. Biol. 1, pp. 854–864). Fifty nanograms of the 1.97 kb BamHI fragment from pIB124/HM1 were ligated with 50 ng of BamHI-digested M13mp18. The ligation was transformed into *E. coli* strain JM103, and Amp<sup>R</sup> colonies were selected. Correct plasmid was identified by the presence of a 1.45 KpnI digestion fragment. Template was prepared from this plasmid to introduce an EcoRI site immediately before the initiation codon of the human HM1 coding region. This was accomplished by standard mutagenesis using the following oligonucleotide to introduce an EcoRI site adjacent (and 5' to) the translation initiation codon of the human HM1 coding region:

5'-CCCCAGCCCC ACCTTGAATT CATGAACACT TCAGCC-3'. (Seq. ID No. 1).

The mutagenesis products were transformed into JM103 and screened on plaque lifts with an oligonucleotide having the sequence:

5'-CACCTTGAAT TCATGAAC-3' (Seq. ID No. 2)

Four of the positive clones were subjected to dideoxy sequencing and all were found to have the correct sequence, i.e., an added EcoRI site immediately 5' of the 5' ATG. One of the positive sequence clones, mHM1AChR103, was selected. Oligonucleotide-directed mutagenesis employing the following oligonucleotide was used to introduce an additional EcoRI site adjacent to (and 3'- to) the translation termination codon of the human HM1 coding sequence:

5'-CTCCCGCCAA TGCTGAGAAT TCTATCTCCT GCATCCC-3' (Seq. ID No. 3)

Mutagenesis products were transformed into JM103 and screened on plaque lifts with an oligonucleotide of the following sequence:

5'-CTGAGAATTCTATCTCC-3' (Seq. ID No. 4). Positive clones were identified and four were sequenced to confirm that the EcoRI site had been introduced and that remainder of the sequence was unaltered. The four sequenced clones had the correct sequence.

One of the sequenced clones, M3HM1AR04, was digested with EcoRI and the 1.4 kb fragment representing the human M1 coding region was gel purified and eluted using DE-81 paper. Sixty nanograms of the 1.4 kb fragment were ligated with 20 ng of EcoRI-digested pUC19. Correct clones were identified by the presence of a 1.2 kb KpnI fragment. One of these was chosen and designated pHM1RO2. The 1.4 kb EcoRI fragment was removed from pHM1RO2 and inserted (38.5 ng) into 50 ng of EcoRI-digested pSV2dhfr. The resulting product was transformed into DH5 $\alpha$  (Sambrook, et al., Molecular Cloning, 2nd ed., Cold Spring Harbor Lab, 1989, p. 10) cells and Amp<sup>R</sup> colonies were selected. Of the selected colonies those that, upon digestion with EcoRI, yielded fragments of 1.4 and 5.0 kb and, upon digestion with PvuII, yielded fragments of 250, 1150, and 5000 bp contained the insert in pSV2dhfr in the correct orientation. The final HM1 expression vector was called HM1pSV2dHFR.

C. Preparation of TK<sup>+</sup> (Thymidine Kinase) Selection Plasmids

Either pThx59 (Zipser, et al. (1981) Proc. Natl. Acad. Sci. 78, pp. 6276–6280), which encodes the wildtype TK gene, or pThx24 (ibid.) which encodes a crippled TK gene was co-transfected into Ltk<sup>-</sup> cells along with the muscarinic receptor-expressing plasmids in order to prepare stably

transfected Ltk cells that express the cloned HM1 receptor on the cell surfaces.

D. Preparation of Stably Transfected Cell Lines Expressing Functional Human M1 Muscarinic Acetylcholine Receptor

Stable HM1-expressing cell lines were prepared using calcium phosphate transfection to introduce the plasmid DNA (see, Wigler, et al. (1979), Proc. Natl. Acad. Sci. 76, pp. 1373–1376). Briefly, Ltk<sup>-</sup> cells were grown in nonselective medium, D+10, which contains Dulbecco's modified Eagle's medium+10% calf serum, in a 10 cm-sized dish, to 20% confluence. The two circular plasmids, the TK<sup>+</sup> plasmid and the HM1-containing plasmid were co-precipitated with CaPO<sub>4</sub> and added to the cell monolayer. The vector concentrations were as follows:

Thx24:HM1pSV2dHFR 2  $\mu$ g:2  $\mu$ g/ml

Thx59:HM1pSV2dHFR 0.25  $\mu$ g:2  $\mu$ g/ml

The final concentration of DNA was adjusted to 20 to 40  $\mu$ g/ml by adding Ltk<sup>-</sup> or pUc DNA. The transfected cells were cultured for two days in nonselective medium. After two days, the cells were passed, non-selective media was replaced with HAT medium (D+10+15  $\mu$ g/ml hypoxanthine+1  $\mu$ g/ml aminopterin+5  $\mu$ g/ml thymidine), and the cells were cultured for 10–15 days, during which time the cells were "fed" fresh selective (HAT) medium every 3–4 days. After 10–15 days, colonies or clones appeared which indicated acceptance and expression of at least the plasmid carrying the TK gene. Colonies were transferred into separate wells of a 24-well dish and grown in selective medium for seven days. Individual clones were then transferred into 6-well dishes and grown for another seven days in selective medium. To provide cells for freezing and subsequent molecular and functional receptor analyses, the individual clones in the 6-well dishes were passed to 100 ml dishes. Two of the resulting cell lines were designated LM124-3 and LM159-10.

## EXAMPLE 2

### Preparation of Recombinant Mammalian Cell Lines by Transfection with cDNAs Encoding Human Neuronal Calcium Channel Subunits

Complementary DNAs encoding  $\alpha$ 1,  $\alpha$ 2 and  $\beta$  subunits of human neuronal voltage-dependent calcium channels (VDCC) were isolated as described in co-owned U.S. patent application Ser. No. 745,206, filed Aug. 15, 1991, the disclosure of which is hereby incorporated by reference. These cDNAs were separately incorporated into mammalian expression vectors for use in recombinant expression of calcium channels in mammalian cells.

#### A. Calcium Channel Subunit Gene Expression Vectors

Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors (which comprises the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria), pCDNA1 or pCMV-based vectors, which comprise the cytomegalovirus (CMV) promoter, or NMTV promoter-based vectors or the vector pCMV.

To construct mammalian expression vectors for use in recombinant expression of human neuronal voltage-dependent calcium channel subunits, portions of partial subunit cDNAs were ligated with PCDNA1 (Invitrogen, San Diego, Calif.) as described in detail in U.S. Ser. No. 745,206. In constructing an  $\alpha$ 1 subunit expression vector, partial  $\alpha$ 1 subunit cDNAs were ligated with EcoRV/XhoI-digested

PCDNA1, and the resulting construct, containing a full-length  $\alpha 1$  subunit-encoding DNA under the regulatory control of the CMV promoter, was called pVDCCIII(A). To construct an  $\alpha 2$  subunit expression vector, portions of partial  $\alpha 2$  subunit cDNAs were ligated with pIBI24 (Stratagene, La Jolla, Calif.) to create HBCaCH $\alpha 2$ . The 3600 bp EcoRI fragment of HBCaCH $\alpha 2$ , which contained the full-length  $\alpha 2$  subunit cDNA, was then subcloned into pCDNA1 to yield pHBCaCH $\alpha 2$ A. Similarly, a mammalian expression vector for use in recombinant expression of a human neuronal calcium channel  $\beta$  subunit was constructed by ligating portions of the  $\beta$  subunit CDNA with pCDNA1. The resulting vector, pHBCaCH $\beta 1$ ba, contained a CDNA encoding a full-length  $\beta$  subunit in which approximately 800 bp of 5' untranslated sequence was replaced with an efficient ribosome binding site (ACCACC).

#### B. Transfection of HEK 293 Cells

HEK 293 cells were transfected using the calcium phosphate transfection procedure [*Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1–9.1.9 (1990)]. Plates (10 cm), each containing one-to-two million HEK 293 cells, were transfected with 1 ml of DNA/calcium phosphate precipitate containing 5  $\mu$ g pVDCCIII(A), 5  $\mu$ g pHBCaCH $\alpha 2$ A, 5  $\mu$ g pHBCaCH $\beta 1$ ba, 5  $\mu$ g pCMVBgal and 1  $\mu$ g pSV2neo (as a selectable marker). After 10–20 days of growth in media containing 500  $\mu$ g G418, colonies had formed and were isolated using cloning cylinders.

### EXAMPLE 3

This example demonstrates the ability of the automated assay to detect agonist and/or antagonist activity of compounds which affect muscarinic acetylcholine receptors, nicotinic acetylcholine receptors or calcium channels.

#### A. Automated Assay for Measuring the Presence and Response of Muscarinic Receptors

Stably transfected muscarinic receptor-expressing LM124-3 cells were plated into 16 wells (2 columns of 8 rows) of a 96-well plastic plate (Costar, Cambridge, Mass.; Catalog No. 3596) at a density of about 250,000 cells per well and incubated overnight at 37° C. in a humidified chamber with 10% CO<sub>2</sub>. Following the incubation, the medium was removed and the plate was washed with 200 microliters per well of HEPES-buffered saline (120 mM NaCl, 5 mM KCl, 0.62 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 6 mM glucose, 10 mM HEPES, pH 7.4). The cells were then loaded with the calcium-sensitive indicator Fluo-3 (Molecular Probes, Inc., Eugene, Oreg.; Catalog No. F1242). Fluo-3 was prepared by reconstituting a 50  $\mu$ g vial to 1 mM by adding 44 microliters dimethylsulfoxide (DMSO). Pluronic F-127, which detergent is used to render the cells permeable to Fluo-3 is included with the Fluo-3 dye reagent. Pluronic F-127 was prepared by dissolving 322 mg of pluronic F-127 in 966  $\mu$ l of DMSO and heating to 45° C. until dissolved to yield a 25% stock solution. 18  $\mu$ l of the 25% pluronic F-127 was added to the reconstituted Fluo-3. A 20  $\mu$ M solution of reconstituted Fluo-3 dye and 0.2% pluronic F-127 was made by adding the contents of the soluble Fluo-3 (including the pluronic F-127 mixture) to 2.2 ml of HEPES-buffered saline (HBS).

25  $\mu$ l of the 20  $\mu$ M Fluo-3/DMSO/pluronic F-127 solution was added to each well of the plates and incubation was carried out for three hours at room temperature in a humidified chamber.

At the end of the three hour incubation, the dye was removed using a multi-channel pipetter and each well was

washed with 180  $\mu$ l of HBS. The fluorometer, Fluoroskan II (ICN Biomedicals, Inc., Horsham, Pa.), as provided by the manufacturer, is capable of reading the 16 wells (columns 1+2) in approximately a 15 second time period not including the lag period for start-up. Because it was desired to read each reaction between about 30 and 50 seconds after the reaction was started with addition of the agonist (carbachol), the plate was placed in the Fluoroskan II unit such that the first well would be read at about 30 seconds after addition of the agonist. The assay was conducted using quadruplicate wells for each treatment. Each set of four wells received 180  $\mu$ l of HBS. In one set of quadruplicate wells, no muscarinic receptor antagonist was added. In the second set of four wells, the HBS was supplemented with 1  $\mu$ M atropine; in the third set of four wells, the HBS was supplemented with 10  $\mu$ M pirenzepine; and in the fourth set of replicate wells the HBS was supplemented with 1  $\mu$ M scopolamine. The plate was incubated at room temperature for three minutes to allow the antagonist compounds to equilibrate with the muscarinic receptors. The background fluorescence was measured. Then, 20  $\mu$ l of 10 mM carbachol (CCh) was added so that the final concentration of carbachol in each well was 1 mM. The 20  $\mu$ l aliquots of carbachol were added with a multiple pipetter and the plate was inserted in the Fluoroskan approximately 20 seconds after addition of the agonist. The plate was read at a rate of about 1 well per second thereafter such that each of the 16 wells were read between 30 and 50 seconds after the reaction was started. The treatments and results were as follows:

Treatment	Fluorescence S/N ratio
1 mM carbachol (CCh)	1.6
1 $\mu$ M atropine + 1 mM CCh	1.0
10 $\mu$ M pirenzepine + 1 mM CCh	1.0
1 $\mu$ M scopolamine + 1 mM CCh	1.0

The results indicate that carbachol induces activation of muscarinic receptors in stably transfected IM124-3 cells which results in an increase in calcium ions in the cytoplasm which is detectable by an increase in fluorescence of the cells. The results also show that the muscarinic receptor antagonists atropine, pirenzepine and scopolamine at the concentrations tested, inhibit muscarinic receptor function. In the absence of muscarinic receptor activation, the fluorescence signal remains substantially at background levels.

A positive control cell line which may be used to measure muscarinic receptor activity (HM1 and/or HM2 forms) is the human neuroblastoma cell line SHSY5Y.

Two columns of a microtiter plate were seeded with SHSY5Y cells at a concentration of 250,000 cells per well and treated essentially as the LM124-3 cells with respect to dye-loading. The measurement of muscarinic receptor function, however, was altered. From the previous experiment it was concluded that the reaction kinetics play a very important role and that more data points were needed soon after drug (e.g., carbachol) addition. Therefore, two major modifications of the assay design were introduced (see Example 4). First, a computer program was developed to take multiple readings from a well.

Second, after software design, testing and incorporation of a liquid handling device (Digiflex) was conducted. Pharmacological analysis was then performed. The wells were measured 5 times before drug addition, then drug was automatically added followed by a 2 second delay, then readings were taken for the next 2 minutes. Typically the

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fluorescence peaked between 5–15 seconds for muscarinic acetylcholine receptors (mAChRs). The peak fluorescence signal-to-noise ratio was determined by dividing the fluorescence signal after drug addition (in the absence of antagonist) by the fluorescence signal before drug addition.

The results were as follows:

Treatment	Fluorescence S/N ratio
1 mM carbachol (CCh)	3.3
1 $\mu$ M atropine + 1 mM CCh	1.0
10 $\mu$ M pirenzepine + 1 mM CCh	1.0
1 $\mu$ M scopolamine + 1 mM CCh	1.0

The results indicate that carbachol induces an increase in intracellular calcium in SHSY5Y cells which is detectable by an increase in fluorescence of the fluorescent dye, Fluo-3. The muscarinic receptors of the SHSY5Y cells may be inhibited by treatment with a muscarinic receptor antagonist such as atropine, pirenzepine or scopolamine. Thus, SHSY5Y cells may serve as assay cells in fluorescence-based assays designed to detect compounds which affect functional muscarinic receptors or as a positive control in the assay.

#### B. Automated Assay for Measuring the Presence and Response of Nicotinic Receptors

The automated assay was used to measure increases in intracellular calcium levels in the IMR32 cell line, a human neuroblastoma cell line which expresses nicotinic acetylcholine receptors (NACHR) (Sher, et al., *J. Neurochem.* 50, pp. 1708–1713 (1988)). The cells were plated and loaded with the fluorescent dye Fluo-3 and the assays were carried out substantially as described in the modified method of Example 3A except that the nicotinic receptors of the cells were activated. Each of the wells contained 180  $\mu$ l of HBS alone or supplemented with one of the following nicotinic receptor inhibitors: 1 nM D-tubocurarine (DTC; Sigma) hexamethonium (Sigma), mecamylamine (Sigma) and atropine (Sigma). The assays were carried out in triplicate wells after the addition to each well of 20  $\mu$ l of 2.0 mM nicotine. The results were as follows:

Treatment	Fluorescence S/N ratio
200 $\mu$ M nicotine	2.3
1 mM DTC + 200 $\mu$ M nicotine	1.0
1 mM hexamethonium + 200 $\mu$ M nicotine	1.0
10 mM mecamylamine + 200 $\mu$ M nicotine	1.0
1 mM atropine + 200 $\mu$ M nicotine	1.0

The results indicate that nicotine induces activation of the nicotinic acetylcholine receptors in IMR32 cells, which can be inhibited by pretreatment of the cells with a nicotinic receptor antagonist, and that the activation of nicotinic receptors may be studied using the automated fluorescence assays of the invention.

#### C. Automated Assay for Measuring the Presence and Response of Calcium Channels

##### 1. Assays of IMR32 and SHSY5Y Cells

The protocol used to evaluate the calcium flux through calcium channels in the cells expressing voltage-dependent calcium channels was similar to that described for assaying nicotinic acetylcholine receptors in Example 4B except that the reaction was initiated by depolarizing the cells with KCl (to 50 mM) and fluorescence measurements were recorded

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as described in Example 4. SHSY5Y cells, induced for six days with 10  $\mu$ M retinoic acid (Sigma), and IMR32 cells, induced for ten days with 1 mM dibutyryl cAMP (dbcAMP) and 2.5  $\mu$ M bromodeoxyuridine (BrdU) (both from Boehringer-Mannheim, Indianapolis, Ind.) were treated with various calcium channel interactive drugs before KCl depolarization and measurement of fluorescence. Induction of SHSY5Y cells and IMR32 cells causes increased expression of functional endogenous calcium channels. The calcium channel agonist, Bay K 8644, which binds to open calcium channels and prolongs their open state, was obtained from Research Biochemicals, Inc., Natick, Mass. The calcium channel antagonists are verapamil (Sigma) and, the inorganic calcium blocker, cadmium (Sigma). The treatments were as follows:

Treatment	Fluorescence Signal to Noise Ratio	
	SHSY5Y	IMR32
50 mM KCl	1.9	2.3
50 mM KCl and 1 $\mu$ M BayK 8644	2.7	2.5
50 mM KCl and 25 $\mu$ M verapamil	1.2	1.7
50 mM KCl and 200 $\mu$ M cadmium	1.0	1.0

The results indicate that KCl induces calcium flux which increases the intracellular concentration of calcium in SHSY5Y and IMR32 cells. The results also show that the calcium flux can be potentiated with Bay K 8644 and inhibited by calcium channel antagonists or blockers. Thus, SHSY5Y and IMR32 cells may be used in fluorescence-based automated assays (e.g., as positive control cells) designed to detect functional calcium channel activity.

##### 2. Assays of Recombinant HEK 293 Cells

HEK 293 cells that had been transfected with cDNAs encoding human neuronal calcium channel  $\alpha$ 1,  $\alpha$ 2 and  $\beta$  (see Example 2), and selected for stable expression were screened using the automated fluorescence-based assay to identify transfectant cell lines that were the most highly responsive to KCl depolarization. The assays were conducted using the fully automated version of the assay, as described for analysis of calcium channels in positive control IMR32 and SHSY5Y cells. Fluorescence was measured prior to addition of KCl to the cells to record background, i.e., noise, fluorescence. Signal fluorescence is the maximal fluorescence recorded after addition of KCl to the cells. The results of the screening were as follows:

CELL LINE	S/N <sup>a</sup>
1old $\alpha$ 1D	1.16 $\pm$ 0.01 (4)
4old $\alpha$ 1D	1.10 $\pm$ 0.05 (4)
8old $\alpha$ 1D	1.33 $\pm$ 0.13 (4)
10old $\alpha$ 1D	1.04 $\pm$ 0.01 (4)
11old $\alpha$ 1D	1.01 $\pm$ 0.01 (4)
12old $\alpha$ 1D	1.38 $\pm$ 0.17 (20)
13old $\alpha$ 1D	1.88 $\pm$ 0.06 (12)
HEK 293 (untransfected)	1.03 $\pm$ .03 (3)

<sup>a</sup>S/N  $\pm$  standard deviation; numbers in parentheses represent the number of assays

These results indicated that cell line 13old expressed the highest level of functional calcium channels. Therefore, this cell line was used in additional automated fluorescence-based assays designed to investigate the pharmacology of the calcium channels expressed in the cells.

Cell line 13old was tested for dose-dependent effects of Bay K 8644 (a dihydropyridine (DHP) which is an agonist of DHP-sensitive calcium channels), and three calcium channel antagonists, verapamil, cadmium and  $\omega$ -CgTx, on KCl-induced fluorescence increases in the cells. The automated assays were performed as described for analysis of calcium channel activity of IMR32 and SHSY5Y cells. For each dose-dependence assay, different wells of the microtiter plate received different concentrations of the agonist or antagonist, the background fluorescence was measured, and KCl was added to a predetermined well via the DigiFlex System. Repeated fluorescence measurements were made before and after KCl addition and the values were recorded. A total of five fluorescence readings were taken before KCl addition. The instrument then moves the plate to the reagent-adding position and 20  $\mu$ l of 0.5M KCl was added to the well (50 mM KCl, final concentration) by the DigiFlex pipetter. The plate was then automatically repositioned under the fluorescence measuring position (a delay of two seconds was measured between reagent addition and the first fluorescent measurement after reagent addition) and 125 fluorescent measurements were taken and values were recorded. Following each assay of a well, another well was assayed until all of the predetermined wells had been assayed. The maximum fluorescence measured for each compound tested and the compound concentration that yielded the maximum fluorescence were noted.

Dose-response curves were generated from the fluorescence data. In constructing these curves, the fluorescence value recorded in the presence of each concentration of compound was plotted as the percent maximum fluorescence vs. concentration. It was possible to estimate  $EC_{50}$  and  $IC_{50}$  values from these curves.

Increasing concentrations of Bay K 8644 potentiated the fluorescence response to KCl in 13old cells, which was maximal at 100 nM. The  $EC_{50}$  for the potentiation of fluorescence response by Bay K 8644 was estimated at 4.4 nM. Verapamil inhibited the fluorescence response with an  $IC_{50}$  of approximately 6.0  $\mu$ M and cadmium with an  $IC_{50}$  of 3.0  $\mu$ M. The antagonist  $\omega$ -CgTx did not completely inhibit the KCl-induced fluorescence increase in 13old cells.

#### EXAMPLE 4

This Example demonstrates the automation of the fluorescent dye-based assays of the invention resulting in increased efficiency in conducting the assays and increased reliability of the results.

The results of the assays of LM124-3 cells presented in Example 3A were obtained by measuring the fluorescence once per well, 20–30 seconds after addition of reagent (i.e., carbachol). Significant variations in the relative amount of fluorescence measured and the calculated signal-to-noise ratios were observed due to the kinetics of the fluorescence reaction. The software controlling the Fluoroskan II was rewritten so as to direct a well of the plate to remain under the excitation/emission head for an extended period of time in order to allow the fluorescence from the well to be measured and recorded repetitively which improved the reliability of the results (i.e., decreased the standard deviation). Using the new program, allowing continuous reading from individual wells, the change in fluorescence over time (i.e., kinetics) could be studied and it was possible to identify a maximal fluorescence response. However, this undesirably limited the number of assays which could be conducted per hour because reagent had to be added to the one well at a time immediately before that well was read in the fluorometer. The average time between addition of

reagent and the first reading was reduced from about 10 seconds to about 6 seconds, but only a single well could be read at one time.

To accomplish rapid drug addition and nearly immediate reading of the fluorescence response, the Fluoroskan II was further modified by fitting a DigiFlex TP automatic pipetter and developing a software program to accomplish precise computer control over both the Fluoroskan II and the DigiFlex TP. By integrating the combination of the Fluoroskan II and the DigiFlex TP and using a microcomputer to control the commands to the fluorometer and automatic pipetter, the delay time between reagent addition and fluorescence reading was reduced from about 6 seconds to about 2 seconds. Moreover, both greater reproducibility and higher signal-to-noise ratios have been achieved as compared to manual addition of reagent since the computer repeats the process precisely time after time. And, extremely advantageously, a plurality of assays may be conducted seriatim, without manual intervention. The results of the fluorescent dye-based assays for muscarinic acetylcholine receptors carried out by manual addition of reagent followed by a single fluorescence measurement (I), manual addition of reagent followed by substantially continuous fluorescence measurements (94 readings per minute) (II) and automatic injection of reagent followed by substantially continuous fluorescence measurements (III) are shown in the table below:

TABLE 4

Summary of Carbachol Induction Data from the Development of the Fluorescent Dye-Based Assay for Muscarinic Acetylcholine Receptors			
		Cell Line	
Assay Development Stage		LM124-3	SHSY5Y
I.	Manual addition of CCh, 30 second delay before single fluorescence measurement	$\overline{S:N} = 1.55$ S.D. = 0.40 (n = 68)	N.D.
II.	Manual addition of CCh, 6 second delay before multiple fluorescence measurements	$\overline{S:N} = 2.0$ S.D. = 0.3 (n = 34)	$\overline{S:N} = 3.3$ S.D. = 0.4 (n = 38)
III.	Automatic injection of CCh, 2 second delay before multiple fluorescence measurements	$\overline{S:N} = 2.12$ S.D. = 0.16 (n = 5)	$\overline{S:N} = 3.62$ S.D. = 0.16 (n = 11)

$\overline{S:N}$  = Average CCh induced signal-to-noise ratio =  $\frac{\text{Fluorescence Signal} + \text{CCh}}{\text{Fluorescence Signal} - \text{CCh}}$   
N.D. = Not Determined  
S.D. = Standard Deviation  
n = number of determinations

Thus, with automatic delivery of reagent followed by multiple fluorescence measurements, reliability of the fluorescent dye-based assay as well as the number of assays that can be performed per day are advantageously increased.

While the invention has been described herein with some specificity, the ordinarily skilled in the art will recognize numerous variations and modifications, in what is described, that are within the spirit of the invention. Such variations and modifications are within the scope of the invention as described in claim herein.

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 create EcoRI site adjacent to initiation codon of  
 human HM1 coding region

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 EcoRI site adjacent to initiation codon of human  
 HM1 coding region

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 HM1 coding region

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What is claimed is:

1. An automated drug screening assay method for identifying compounds that modulate the activity of ion channels and/or receptors of a cell, comprising:

(a) introducing a divided culture vessel that has an array of individual compartments into an automated assay apparatus, wherein:

the apparatus delivers reagent solution to one or more of the compartments and has detection means to measure transient changes in an optical attribute in the compartments;

at least two of the compartments contain viable cells that comprise either or both the ion channel and receptor; at least one, and up to one fewer than all of the cell-containing compartments also comprises one or more test compound(s) that is (are) being screened for the ability to modulate the activity of the ion channel or receptor;

the ion channel or receptor, when activated, directly or indirectly contributes to a detectable change in the cytoplasmic level of a predetermined ion in the cells; and



the cytoplasm of the cells contains an amount of an ion-sensitive fluorescent indicator sufficient to detect a change in the concentration of the predetermined ion;

(b) automatically delivering to a cell-containing compartment, an aliquot of a solution comprising an amount of a known ion channel- or receptor-activating compound that activates the ion channel or receptor, whereby, the change in concentration of the predetermined ion commences;

(c) within a time period before any induced ion concentration change would be at a maximum, automatically detecting and measuring for a predetermined amount of time in the cell-containing compartment to which an aliquot of solution has been added ion flux by measuring fluorescence emitted by the ion-sensitive indicator in response to an excitation wavelength, whereby compounds that modulate the activity of ion channels and/or receptors of a cell are identified.

2. An automated drug screening assay of claim 1, wherein the ion channels or receptors are capable of fluxing said ion and the cells are bathed in a solution comprising a concentration of said ions which is sufficient to cause a detectable increase in the level of said ions in the cytoplasm when said ion channels or receptors are activated.

3. An automated drug screening assay according to claim 2 wherein said cells comprise voltage-dependent calcium channels and the solution bathing the cells comprises a concentration of calcium ions which is sufficient to cause a detectable increase in the level of calcium ions in the cytoplasm when the calcium channels are activated by membrane depolarization.

4. An automated drug screening assay according to claim 3 wherein said cells are recombinant cells which express heterologous calcium channels.

5. An automated drug screening assay according to claim 4 wherein said recombinant cells are transfected human embryonic kidney cells.

6. An automated drug screening assay according to claim 2 wherein said cells have ligand-gated ion channels.

7. An automated drug screening assay of claim 3, wherein:

the cells have both voltage-dependent calcium channels and ligand-gated ion channels, and

the known receptor-activating compound is a ligand capable of activating the ligand-gated ion channel and depolarizing the cell membranes whereby the voltage-dependent calcium channels open.

8. An automated drug screening assay of claim 7, wherein the test compound is screened for its ability to inhibit activation of the ligand-gated ion channels.

9. An automated drug screening assay of claim 1, wherein the cells comprise a G-protein-coupled receptor and the known receptor-activating compound is a ligand capable of activating the receptor so as to cause an increase in the level of cytoplasmic calcium ions.

10. An automated drug screening assay of claim 9, wherein the G-protein-coupled receptors is selected from the group consisting of metabotropic EAA receptors and muscarinic acetylcholine receptors.

11. The assay of claim 6, wherein the cells comprise ligand-gated ion channels comprising a nicotinic acetylcholine receptor, kainate/AMPA receptor, or a nicotinic acetylcholine receptor and a kainate/AMPA receptor.

12. The method of claim 1, further comprising, after step (c) comparing the intensity of fluorescence in a cell-containing compartment comprising the test compound with the intensity of fluorescence produced by a substantially

identical cell-containing compartment treated substantially identically except in that it does not contain the test compound.

13. The method of claim 1, wherein steps (b) and (c), are repeated until all of the cell-containing compartments have been measured.

14. The method of claim 12, wherein steps (b) and (c) are repeated until all of the cell-containing compartments have been measured.

15. The method of claim 1, further comprising (d) repeating steps (b) and (c) successively for one or more of the remaining cell-containing compartments.

16. The method of claim 1, wherein the time period between steps (b) and (c) is less than or equal to about 30 seconds.

17. An automated drug screening assay of claim 3, wherein the cells are Ltk<sup>-</sup> cells, COS-7 cells, DG44 cells, or Chinese hamster ovary (CHO) cells.

18. The method of claim 1, wherein the test compound activates the ion channel or receptor.

19. The method of claim 1, wherein the test compound antagonizes the activity of the ion channel or receptor.

20. The method of claim 1, wherein the test compound potentiates the activity of the ion channel or receptor.

21. An automated drug screening assay method for identifying compounds that modulate the activity of ion channels and/or receptors of a cell, comprising:

(a) introducing a divided culture vessel that has an array of individual compartments into an automated assay apparatus, wherein:

the apparatus delivers reagent solution to one or more of the compartments and has detection means to measure transient changes in an optical attribute in the compartments;

at least two of the compartments contain viable cells, wherein the cells comprise the ion channel or receptor; the ion channel or receptor, when activated, directly or indirectly contributes to a detectable change in the cytoplasmic level of a predetermined ion in the cells; and

the cytoplasm of the cells contain an amount of an ion-sensitive fluorescent indicator sufficient to detect a change in the concentration of the predetermined ion;

(b) automatically delivering to a cell-containing compartment, an aliquot of a solution comprising a test compound being tested for ability to alter the activity of the ion channel or receptor, whereby, if the test compound activates the ion channel or receptor, a change in the concentration of the predetermined ion commences;

(c) within a time period before any concentration change of the predetermined ion would be at its maximum, automatically detecting and measuring in one or more of the cell-containing compartment to which the aliquot of solution has been added, for a predetermined amount of time, ion flux by measuring the fluorescence emitted by the ion-sensitive indicator in response to an excitation wavelength, whereby compounds that modulate the activity of ion channels and/or receptors of a cell are identified.

22. An automated drug screening assay of claim 21, wherein the ion channels or receptors are capable of fluxing said ion and the cells are bathed in a solution comprising a concentration of said ions which is sufficient to cause a detectable increase in the level of said ions in the cytoplasm when said ion channels or receptors are activated.

23. An automated drug screening assay according to claim 22 wherein said cells comprise voltage-dependent calcium

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channels and the solution bathing the cells comprises a concentration of calcium ions which is sufficient to cause a detectable increase in the level of calcium ions in the cytoplasm when the calcium channels are activated by membrane depolarization.

24. An automated drug screening assay according to claim 23 wherein said cells are recombinant cells which express heterologous calcium channels.

25. An automated drug screening assay according to claim 24 wherein said recombinant cells are transfected human embryonic kidney cells.

26. An automated drug screening assay according to claim 22 wherein said cells have ligand-gated ion channels.

27. An automated drug screening assay of claim 23, wherein said cells have both voltage-dependent calcium channels and ligand-gated ion channels, and wherein, activation of the ligand-gated ion channel depolarizes the cell membrane so as to open the voltage-dependent calcium channels.

28. An automated drug screening assay of claim 21, further comprising in step (b) adding a known activator of the ion channel or receptor simultaneously with or before addition of the test compound, wherein the test compound is being screened for its ability to inhibit activation of the ion channel or receptor.

29. An automated drug screening assay of claim 21, wherein: the cells comprise a G-protein-coupled receptor; and activation of the receptor causes an increase in the level of an ion in the cytoplasm.

30. An automated drug screening assay according to claim 29 wherein the cells comprise receptors selected from the group consisting of metabotropic EAA receptors and muscarinic acetylcholine receptors.

31. A method according to claim 27 wherein the cells comprise a receptor selected from the group consisting of nicotinic acetylcholine receptors and kainate/AMPA receptors.

32. The method of claim 21, further comprising repeating steps (b) and (c) successively for one or more of the remaining cell-containing compartments.

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33. The method of claim 21, further comprising:

(d) comparing the intensity of fluorescence in the cell-containing compartments that received the test compound with the intensity of fluorescence produced by one or more of cell-containing compartments that received a solution that is devoid of any compound that activates the ion channels or receptors.

34. The method of claim 21, wherein steps (b) and (c) are repeated until all of cell-containing compartments have been measured.

35. The method of claim 33, wherein steps (b) and (c) are repeated until all of cell-containing compartments have been measured.

36. The method of claim 21, further comprising, after step (d) comparing the intensity of fluorescence in a cell-containing compartment that received the test compound with the intensity of fluorescence produced by a substantially identical cell-containing compartment treated substantially identically except that the cells do not comprise the receptor or ion channel.

37. The method of claim 21, wherein the time period between steps (b) and (c) is less than or equal to about 30 seconds.

38. An automated drug screening assay of claim 22, wherein the cells are Ltk<sup>-</sup> cells, COS-7 cells, DG44 cells, or Chinese hamster ovary (CHO) cells.

39. The method of claim 21, wherein the test compound activates the ion channel or receptor.

40. The method of claim 21, wherein the test compound antagonizes the activity of the ion channel or receptor.

41. The method of claim 21, wherein the test compound potentiates the activity of the ion channel or receptor.

42. The method of claim 21, wherein prior to adding the test compound, an agonist of the ion channel or receptor is added to one or more cell-containing compartments.

43. The method of claim 40, wherein prior to adding the test compound, an agonist of the ion channel or receptor is added to one or more cell-containing compartments.

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